

(19)

Europäisches Patentamt  
European Patent Office  
Office européen des brevets



(11)

**EP 1 130 042 A2**

(12)

**EUROPEAN PATENT APPLICATION**

(43) Date of publication:  
05.09.2001 Bulletin 2001/36

(51) Int Cl.<sup>7</sup>: **C08G 63/06, C12P 7/62**

(21) Application number: **01104925.1**

(22) Date of filing: **28.02.2001**

(84) Designated Contracting States:  
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU  
MC NL PT SE TR**  
Designated Extension States:  
**AL LT LV MK RO SI**

(30) Priority: **29.02.2000 JP 2000054668**  
**29.02.2000 JP 2000054669**  
**27.09.2000 JP 2000294634**

(71) Applicant: **CANON KABUSHIKI KAISHA**  
**Tokyo (JP)**

(72) Inventors:  
• **Honma, Tsutomu**  
**Ohta-ku, Tokyo (JP)**

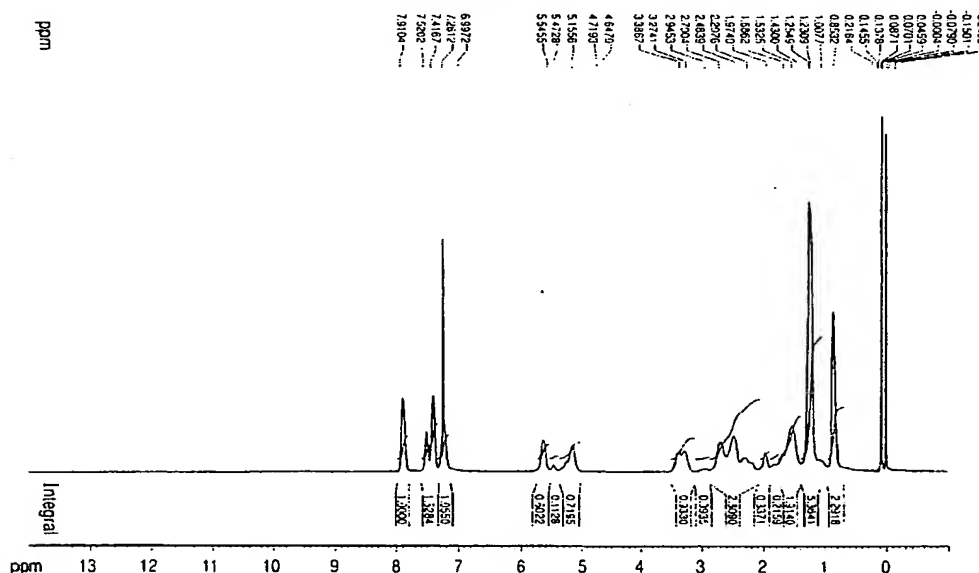
- **Sugawa, Etsuko**  
**Ohta-ku, Tokyo (JP)**
- **Yano, Tetsuya**  
**Ohta-ku, Tokyo (JP)**
- **Kobayashi, Shin**  
**Ohta-ku, Tokyo (JP)**
- **Imamura, Takeshi**  
**Ohta-ku, Tokyo (JP)**
- **Kenmoku, Takashi**  
**Ohta-ku, Tokyo (JP)**

(74) Representative: **Weser, Wolfgang, Dr. Dipl.-Phys.**  
**Weser & Kollegen,**  
**Patentanwälte,**  
**Radeckestrasse 43**  
**81245 München (DE)**

(54) **Polyhydroxyalkanoate containing 3-hydroxybenzoylalkanoic acid as monomer unit, and method for producing the same**

(57) Microorganisms capable of producing novel polyalkanoates having 3-hydroxybenzoylalkanoic acids as monomer units by utilizing benzoylalkanoic acids as

starting materials are cultured in media containing benzoylalkanoic acids and saccharides, and the polyhydroxyalkanoates produced in the culture cells are extracted and recovered.

**FIG. 1****Best Available Copy****EP 1 130 042 A2**

## Description

## BACKGROUND OF THE INVENTION

## Field of the Invention

**[0001]** The present invention relates to a novel polyhydroxyalkanoate (hereinafter, sometimes abbreviated to "PHA"). Further, the present invention relates to a method for high efficient production of PHA using microorganisms having an ability of producing PHA and accumulating it in their cells.

## Related Background Art

**[0002]** Heretofore, a variety of microorganisms have been reported to produce and accumulate poly-3-hydroxybutyric acid (hereinafter, sometimes abbreviated to "PHB") or other PHAs in the cells ("Biodegradable Plastics Handbook", edited by Biodegradable Plastics Society, issued by NTS Co., Ltd., P178-197). These polymers can be used for production of different kinds of products with melting processes etc. like the conventional plastics. Further, since these polymers have the advantage of being completely decomposed by microorganisms in nature because of their biodegradability, they would not remain in the natural environment to cause pollution unlike many conventional synthetic polymer compounds. Furthermore, they are also excellent in biological compatibility and expected to be applied to medical soft members and the like.

**[0003]** It is known that such microbial PHAs may have a variety of compositions and structures depending on types of the microorganisms, the compositions of culture media, culture conditions, etc. used for their production, and up to now, studies regarding the control of these compositions and structures have been carried out from the point of view of improving the properties of PHAs.

**[0004]** For example, it has been reported that *Alcaligenes eutropus* strain H16 (ATCC No. 17699) and its mutant strains produce copolymers of 3-hydroxybutyric acid (hereinafter, sometimes abbreviated to "3HB") and 3-hydroxyvaleric acid (hereinafter, sometimes abbreviated to "3HV") at a variety of composition ratios by varying the carbon sources in their culture (Japanese Patent Publication Nos. 6-15604, 7-14352, 8-19227 and the like).

**[0005]** Japanese Patent Application Laid-Open No. 5-74492 discloses a method in which the copolymer of 3HB and 3HV is produced by bringing *Methylobacterium* sp., *Paracoccus* sp., *Alcaligenes* sp. or *Pseudomonas* sp. into contact with primary alcohol having 3 to 7 carbons.

**[0006]** Japanese Patent Application Laid-Open Nos. 5-93049 and 7-265065 disclose that two-component copolymers of 3HB and 3-hydroxyhexanoic acid (hereinafter, sometimes abbreviated to "3HHx") are produced by culturing *Aeromonas caviae* using oleic acid or olive oil as a carbon source.

**[0007]** Japanese Patent Application Laid-Open No. 9-191893 discloses that *Comamonas acidovorans* IFO 13852 produces polyester having 3HB and 4-hydroxybutyric acid as monomer units in culture with gluconic acid and 1, 4-butanediol as a carbon source.

**[0008]** Also, in recent years, intensive researches about PHA composed of 3-hydroxyalkanoate (hereinafter, sometimes abbreviated to "3HA") of medium-chain-length (abbreviated to "mcl") having up to about 12 carbons have been performed. Synthetic routes of such PHAs can be classified broadly into two types, and their specific examples will be shown in (1) and (2) below.

(1) Synthesis using  $\beta$ -oxidation

**[0009]** Japanese Patent No. 2642937 discloses that PHA having monomer units of 3-hydroxyalkanoate having 6 to 12 carbons is produced by giving a noncyclic aliphatic hydrocarbon as a carbon source to *Pseudomonas oleovorans* ATCC 29347.

**[0010]** Furthermore, it has been reported in Appl. Environ. Microbiol., 58 (2), 746 (1992) that *Pseudomonas resinovorans* produces polyester having 3-hydroxybutyric acid, 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid and 3-hydroxydecanoic acid (in a quantitative ratio of 1:15:75:9) as monomer units, using octanoic acid as a sole carbon source, and also produces polyester having 3-hydroxybutyric acid, 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid and 3-hydroxydecanoic acid (in a quantitative ratio of 8:62:23:7) as units, using hexanoic acid as a sole carbon source. Herein, it is assumed that 3HA monomer units having a chain length longer than that of fatty acids as a starting material are made by way of a fatty acid synthetic route that will be described in (2).

## (2) Synthesis using fatty acid synthetic route

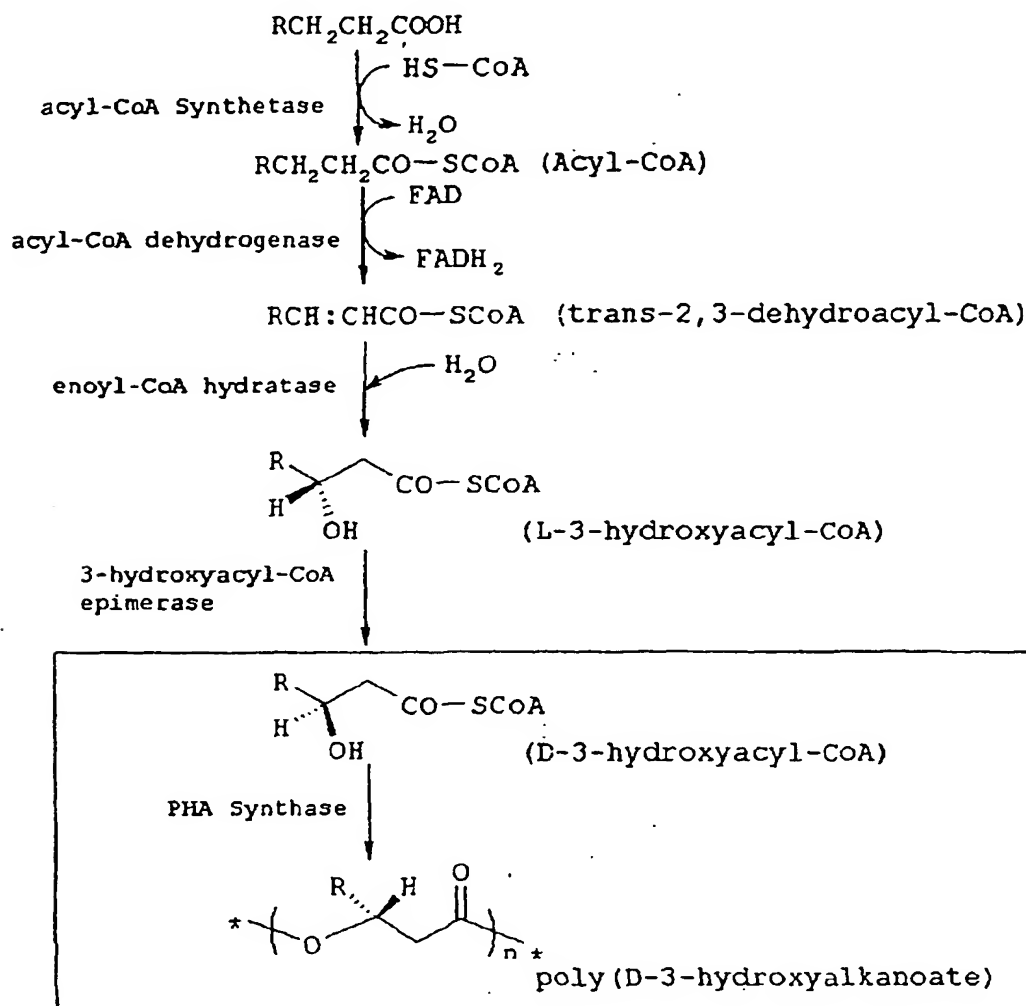
**[0011]** It has been reported in Int. J. Biol. Macromol., 16 (3), 119 (1994) that *Pseudomonas* sp. strain 61-3 produces

polyester composed of 3-hydroxyalkanoic acids such as 3-hydroxybutyric acid, 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid; and 3-hydroxyalkenoic acids such as 3-hydroxy-5-cis-decenoic acid and 3-hydroxy-5-cis-dodecenoic acid as units, using sodium gluconate as a sole carbon source.

[0012] By the way, the biosynthesis of PHA is usually carried out by a PHA synthase using "D-3-hydroxyacyl-CoA", which is formed as an intermediate of a variety of metabolic pathways in the cell, as a substrate.

[0013] Herein, "CoA" means a "coenzyme A". As described in the prior art of the above (1), the biosynthesis of PHA is carried out using "D-3-hydroxyacyl-CoA" formed in the "β-oxidation cycle" as a starting substance in the case where fatty acids such as octanoic acid and nonanoic acid are used as carbon sources.

[0014] Reactions through which PHA is biosynthesized by way of the "β-oxidation cycle" will be shown below.



[0015] On the other hand, as described in the prior art of the above (2), in the case where the PHA is biosynthesized using saccharides such as glucose, it is carried out using "D-3-hydroxyacyl-CoA", which is converted from "D-3-hydroxyacyl-ACP" formed in the "fatty acid synthesis pathway", as a starting substance.

[0016] Herein, "ACP" means an "acyl carrier protein".

[0017] By the way, as described previously, any PHA synthesized in both (1) and (2) described above is PHA composed of monomer units having alkyl groups in side chains, that is, "usual PHA". However, if a wider range of application of the microbial PHA like this, for example an application as a functional polymer is considered, it is expected that PHAs having substituents (e.g., phenyl groups) other than alkyl groups introduced in the side chains are significantly useful. Such other substituents include an unsaturated hydrocarbon, ester group, allyl group, cyano group, halogenated hydrocarbon, epoxide or the like.

[0018] With respect to the synthesis of PHA having such substituent (that is, other than alkyl groups) introduced in

the side chain (hereinafter, referred to as "unusual PHA" if necessary), the synthesis using  $\beta$ -oxidation has been reported, for example, a report regarding PHA having an aryl group and the like introduced in the side chain can be found in *Macromolecules*, 24, p5256-5260 (1991). Specifically, it has been reported that *Pseudomonas oleovorans* produces PHA comprising 3HV, 3-hydroxyheptanoic acid, 3-hydroxynonanoic acid, 3-hydroxyundecanoic acid and 3-hydroxy-5-phenylvaleric acid (hereinafter, sometimes abbreviated to "3HPV") in a quantitative ratio of 0.6:16.0:41.1:1.7:40.6 as monomer units in the amount of 160 mg per liter (L) of culture solution (ratio in dry weight to the cell mass is 31.6%), using 5-phenylvaleric acid (hereinafter, sometimes abbreviated to "PVA") and nonanoic acid (mole ratio of 2:1, total concentration of 10 mmol/L) as substrates, and also produces PHA comprising 3HHx, 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid and 3HPV in a quantitative ratio of 7.3:64.5:3.9:24.3 as monomer units in the amount of 200 mg per L of culture solution (ratio in dry weight to the cell mass is 39.2%), using PVA and octanoic acid (mole ratio of 1:1, total concentration of 10 mmol/L) as substrates. It is assumed that the PHA in this report is synthesized through mainly the  $\beta$ -oxidation pathway because nonanoic acid and octanoic acid are used.

[0019] The related description is also found in *Macromol. Chem.*, 191, 1957-1965 (1990) and *Chirality*, 3, 492-494 (1991) where changes of polymer properties presumably caused by containing 3HPV are recognized.

[0020] As described above in microbial PHAs, different compositions and structures can be obtained by changing types of microorganisms, the compositions of culture medium, culture conditions and the like which are used for their production, however, when considering their application to plastics, their properties can not be said to be satisfactory yet. In order to further expand the usable range of microbial PHAs, it is important to investigate wider improvement of the properties, and therefore it is essential to develop and search PHAs comprising monomer units having a variety of structures, production methods therefor, and microorganisms capable of efficiently producing the desired PHAs.

[0021] On the other hand, as described previously, the PHA (unusual PHA) having substituents introduced in the side chain can be also expected to be developed as a "functional polymer" provided with significantly useful functions and characteristics owing to characteristics etc. of the introduced substituents by selecting substituents to be introduced corresponding to the desired characteristics, therefore it is an important problem to develop and search superior PHAs having both such functionality and biodegradability compatible with each other, production methods therefor, and microorganisms capable of efficiently producing the desired PHAs.

[0022] Other examples of PHAs having such substituents introduced in the side chain include PHAs having the above described phenyl group and further phenoxy group in the side chain.

[0023] For another examples of the phenyl group, it has been reported in *Macromolecules*, 29, 1762-1766 (1996) that *Pseudomonas oleovorans* produces PHA comprising 3-hydroxy-5-(4-tolyl)valeric acid as monomer units by culturing in the culture medium containing 5-(4-tolyl)valeric acid (5-(4-methylphenyl)valeric acid) as a substrates.

[0024] Further, it has been reported in *Macromolecules*, 32, 2889-2895 (1999) that *Pseudomonas oleovorans* produces PHA comprising 3-hydroxy-5-(2, 4-dinitrophenyl)valeric acid and 3-hydroxy-5-(4-nitrophenyl)valeric acid as monomer units by culturing in the culture medium containing 5-(2, 4-dinitrophenyl)valeric acid and nonanoic acid as substrates.

[0025] In addition, for an example of the phenoxy group, it has been reported in *Macromol. Chem. Phys.*, 195, 1665-1672 (1994) that *Pseudomonas oleovorans* produces PHA comprising 3-hydroxy-5-phenoxyvaleric acid and 3-hydroxy-9-phenoxyundecanoic acid as units from 11-phenoxyundecanoic acid.

[0026] Further, it has been reported in *Macromolecules*, 29, 3432-3435 (1996) that *Pseudomonas oleovorans* is used to produce PHAs comprising 3-hydroxy-4-phenoxybutyric acid and 3-hydroxy-6-phenoxyhexanoic acid as units from 6-phenoxyhexanoic acid; PHAs comprising 3-hydroxy-4-phenoxybutyric acid, 3-hydroxy-6-phenoxyhexanoic acid and 3-hydroxy-8-phenoxyoctanoic acid as units from 8-phenoxyoctanoic acid; and PHAs comprising 3-hydroxy-5-phenoxybutyric acid and 3-hydroxy-7-phenoxyheptanoic acid as units from 11-phenoxyundecanoic acid, respectively. The yields of polymers cited from this report are as follows.

Table 1

Carbon Source (alkanoate)	Weight of Dry Cell (mg/L)	Weight of Dry Polymer (mg/L)	Yield (%)
6-Phenoxyhexanoic acid	950	100	10.5
8-Phenoxyoctanoic acid	820	90	11
11-Phenoxyundecanoic acid	150	15	10

[0027] Furthermore, in *Can. J. Microbiol.*, 41, 32-43 (1995), PHA is successfully produced which comprises 3-hydroxy-p-cyanophenoxyhexanoic acid or 3-hydroxy-p-nitrophenoxyhexanoic acid as monomer units, using octanoic acid and p-cyanophenoxyhexanoic acid or p-nitrophenoxyhexanoic acid as substrates and using *Pseudomonas oleovorans* strain ATCC29347 and *Pseudomonas putida* strain KT2442.

[0028] In Japanese Patent No. 2989175, homopolymers composed of a 3-hydroxy-5-(monofluorophenoxy)pen-

tanoate (3H5(MFP)P) unit or 3-hydroxy-5-(difluorophenoxy)pentanoate (3H5(DFP)P) unit, and copolymers comprising at least the 3H5(MFP)P unit or 3H5(DFP)P unit; *Pseudomonas putida* for synthesizing these polymers; and methods of producing the aforementioned polymers using the genus *Pseudomonas*.

[0029] These productions are carried out in the following "two-step culture".

Culture time: 24 hours for first step; 96 hours for second step

[0030] Substrates for each step and polymers obtained are shown below.

(1) Polymer obtained: 3H5(MFP)P homopolymer

Substrate for first step: citric acid, yeast extract

Substrate for second step:

monofluorophenoxyundecanoic acid

(2) Polymer obtained: 3H5(DFP)P homopolymer

Substrate for first step: citric acid, yeast extract

Substrate for second step:

difluorophenoxyundecanoic acid

(3) Polymer obtained: 3H5(MFP)P copolymer

Substrate for first step: octanoic acid or nonanoic acid, yeast extract

Substrate for second step:

monofluorophenoxyundecanoic acid

(4) Polymer obtained: 3H5(DFP)P copolymer

Substrate for first step: octanoic acid or nonanoic acid, yeast extract

Substrate for second step:

difluorophenoxyundecanoic acid

[0031] As the effect, it is described that polymers having phenoxy groups substituted with one to two fluorine atoms in the terminal side chain can be synthesized by assimilating medium-chain fatty acids having substituents, and that stereoregularity and water-repellency can be added to them while maintaining a high melting point and good workability.

[0032] In addition, PHAs containing a cyclohexyl group in monomer units are expected to show polymer properties different from those of PHAs containing usual aliphatic hydroxyalkanoic acids as units, and an example of their production by *Pseudomonas oleovorans* has been reported (Macromolecules, 30, 1611-1615 (1997)).

[0033] According to this report, when culturing *Pseudomonas oleovorans* in the medium where nonanoic acid (hereinafter, abbreviated to "NA") coexists with cyclohexylbutyric acid (hereinafter, abbreviated to "CHBA") or cyclohexylvaleric acid (hereinafter, abbreviated to "CHVA"), the PHAs containing units of the cyclohexyl group and units derived from the nonanoic acid are obtained (each ratio is unknown).

[0034] For the yield etc., it has been reported that the quantitative ratios of CHBA and NA were changed in the condition of 20 mmol/L of the total substrate concentration with respect to CHBA to obtain the results as shown in Table 2.

Table 2

NA : CHBA	CDW	PDW	Yield	Unit
5 : 5	756.0	89.1	11.8	NA, CHBA
1 : 9	132.8	19.3	14.5	NA, CHBA
CDW: Cell (Dry Weight) (mg/L) PDW: Polymer (Dry Weight) (mg/L) Yield: PDW/CDW (%)				

[0035] However, the polymer yield per culture solution is insufficient in this case, and aliphatic hydroxyalkanoic acids derived from nonanoic acid are also mixed in the monomer units of the PHA itself obtained.

[0036] Thus, when producing PHAs with various substituents introduced in the side chain using microorganisms, as

seen in the aforementioned report examples of *Pseudomonas oleovorans*, a method is used in which alkanoates having substituents to be introduced are used as carbon sources for growth, in addition to being used as polymer ingredients.

[0037] However, the method in which alkanoates having substituents to be introduced are used as carbon sources for growth, in addition to being used as polymer ingredients are expected to supply an energy source based on generation of acetyl-CoA by way of the  $\beta$ -oxidation from the above-described alkanoates. In such methods, the acetyl-CoA can not be generated by the  $\beta$ -oxidation unless the substrate has a chain-length to a certain extent, therefore it is a significant problem that alkanoates usable as substrates of PHAs are limited.

[0038] Further generally, substrates each having a chain-length shortened by two-methylene chains each through the  $\beta$ -oxidation are newly formed and are incorporated as monomer units of PHAs, therefore the synthesized PHAs often become copolymers comprising monomer units each having a different chain length by two-methylene chains. In the report described above, the copolymers are produced which are composed of 3 types of monomer units: 3-hydroxy-8-phenoxyoctanoic acid derived from 8-phenoxyoctanoic acid as a substrate, 3-hydroxy-6-phenoxyhexanoic acid and 3-hydroxy-4-phenoxybutyric acid which are byproducts derived from the metabolites.

[0039] When intending to obtain PHA composed of a single monomer unit, it is extremely difficult to use this method from this point of view. Further, in the method provided that supply of an energy source is based on the generation of acetyl-CoA by  $\beta$ -oxidation, it is a significant problem that growth of microorganisms is slow and syntheses of PHAs take long time and that the yield of synthesized PHAs is likely to lower.

[0040] Therefore, in addition to alkanoates having substituents intended to be introduced, generally the method is used which is presumably effective in extracting PHAs after culturing microorganisms in the medium coexisting with medium-chain-fatty acids such as octanoic acid and nonanoic acid as carbon sources for growth.

[0041] However, according to the present inventors' consideration, the PHAs synthesized through the  $\beta$ -oxidation pathway where medium-chain-fatty acids such as octanoic acid and nonanoic acid as described above are used as carbon sources for growth have the low purity, and 50% of polymers obtained or more is mcl-3HA monomer units, that are, "usual PHA" units, which are monomer units (e.g., 3-hydroxyoctanoic acid, 3-hydroxynonanoic acid, etc.) derived from carbon sources for growth. These mcl-3HA units are adhesive polymers in single composition at an ordinary temperature, and when mcl-3HA units are mixed in large amounts with the objective PHAs of the present invention, they significantly lower the glass transition temperature ( $T_g$ ) of the polymers.

[0042] Thus, when obtaining a hard polymer property at an ordinary temperature, a mixture of the mcl-3HA monomer units is undesirable. It is known also that such a heterogeneous side chain structure interferes with the interaction derived from intramolecular or intermolecular side chain structures so that it significantly affects crystallinity or orientation. When attaining improvement of the polymer property and addition of functionality, a mixture of the mcl-3HA monomer units is significantly problematic.

[0043] A solution of the problem is to have a purification step for separating/removing "unobjective" monomer units such as mcl-3HA monomer units derived from a carbon source for growth in order to obtain PHAs composed of only a monomer unit having a specified substituent. However, there are problems that it complicates the operation and can not avoid significant reduction of the yield.

[0044] There is also a significant problem that it is extremely difficult to remove only unobjective monomers when forming copolymers from the objective and unobjective monomer units. Particularly, when aiming at the syntheses of PHAs containing monomer units having a side chain such as a group obtained from an unsaturated hydrocarbon, ester group, allyl group, cyano group, nitro group or a group obtained from a halogenated hydrocarbon, a group introduced with epoxide or the like as the side chain structure, the mcl-3HA monomer units often form a copolymer with the objective monomer unit so that it is extremely difficult to remove the mcl-3HA monomer units after synthesis of the PHAs.

## SUMMARY OF THE INVENTION

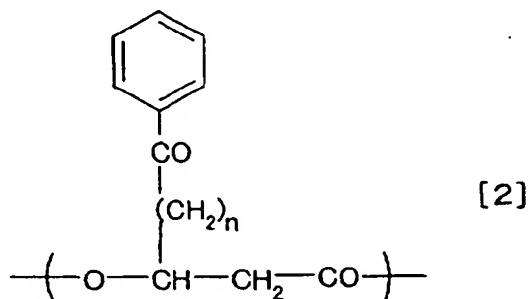
[0045] Therefore, the present inventors have attained the recognition that development of a biosynthetic method for obtaining "unusual PHAs" in high purity is essential, in the case of considering application to functional polymers. Thus, it was presumably highly useful and important to develop excellent polymers provided with both functionality and biodegradability as described above, microorganisms capable of producing the present polymers and accumulating them in the cells, and a method for efficiently biosynthesizing the present polymers in high purity.

[0046] The present invention solves the above-described problems; and provides PHAs (unusual PHAs) comprising monomer units of various structures having substituents in the side chains useful as device materials, medical materials and the like; and provides a method for producing the present "unusual PHAs" using microorganisms, particularly a production method in which the mixture of unobjective monomer units is reduced and an objective "unusual PHA" can be obtained with a high purity and a high yield.

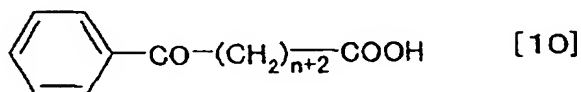
[0047] Accordingly, while the present inventors aim at development of PHAs having substituents in the side chain which are useful as device materials, medical materials and the like, they have searched microorganisms capable of producing different types of PHAs and accumulating them in the cells, and assiduously repeated studies on a method

for producing the desired PHAs using such microorganisms.

[0048] As a result, the present inventors found that microorganisms capable of producing novel PHAs comprising 3-hydroxybenzoylalkanoic acid represented by the following Chemical Formula [2] as a monomer unit and accumulating them in the cells, further that the PHAs can be biosynthesized by culturing these microorganisms under coexisting of benzoylalkanoic acids represented by the following Chemical Formula [10] with saccharides, and that the PHAs obtained thereby had a relatively high purity.

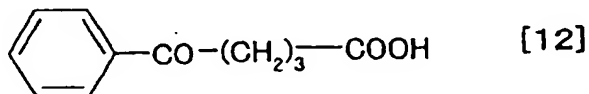


wherein n is any integer of 1 to 8.

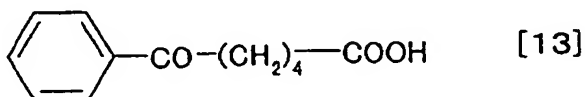


wherein n is any integer of 1 to 8.

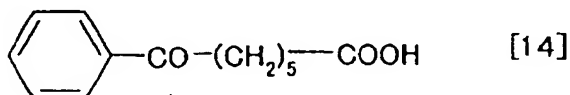
More specifically, the present inventors found microorganisms capable of using as a starting material, 4-benzoylbutyric acid (hereinafter, sometimes abbreviated to "BzBA") represented by the Chemical Formula [12]:



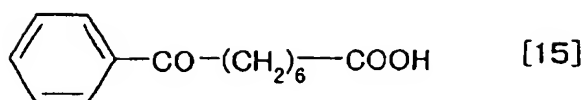
5-benzoylvaleric acid (hereinafter, sometimes abbreviated to "BzVA") represented by the Chemical Formula [13]:



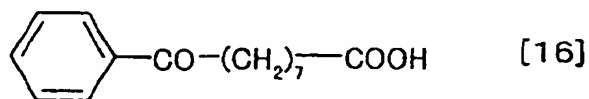
6-benzoylhexanoic acid (hereinafter, sometimes abbreviated to "BzHxA") represented by the Chemical Formula [14]:



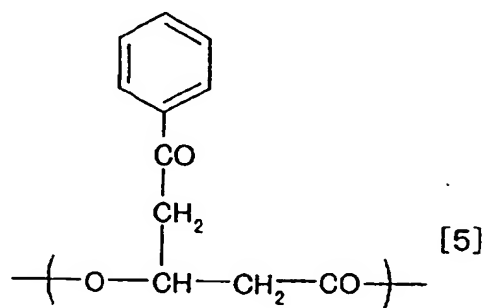
7-benzoylheptanoic acid (hereinafter, sometimes abbreviated to "BzHpA") represented by the Chemical Formula [15]:



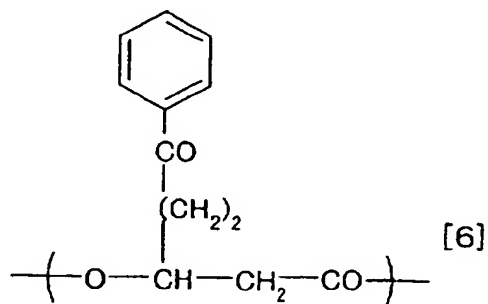
and 8-benzoyloctanoic acid (hereinafter, sometimes abbreviated to "BzOA") represented by the Chemical Formula [16]:



and capable of producing and accumulating in the cells the novel PHAs containing as monomer units, 3-hydroxy-4-benzoylbutyric acid (hereinafter, sometimes abbreviated to "3HBzB") represented by the Chemical Formula [5]:

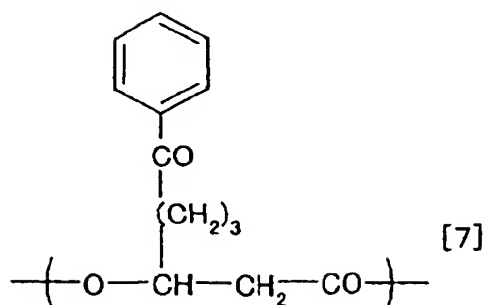


3-hydroxy-5-benzoylvaleric acid (hereinafter, sometimes abbreviated to 3HBzV) represented by the Chemical Formula [6]:

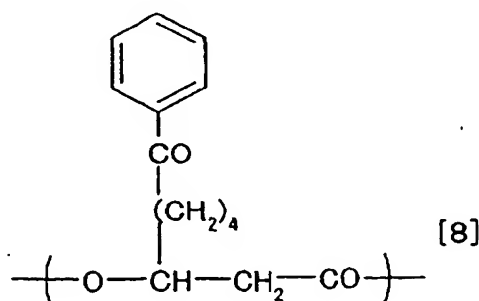


3-hydroxy-6-benzoylhexanoic acid (hereinafter, sometimes abbreviated to "3HBzHx") represented by the Chemical Formula [7]:

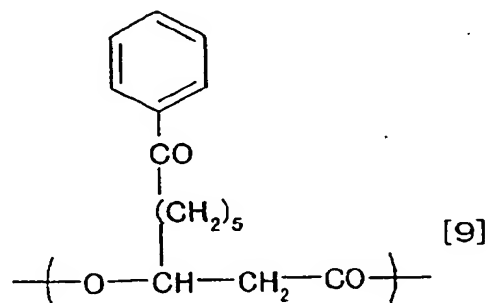




15 and 3-hydroxy-7-benzoylheptanoic acid (hereinafter, sometimes abbreviated to "3HBzHp") represented by the Chemical Formula [8]:



30 3-hydroxy-8-benzoyloctanoic acid (hereinafter, sometimes abbreviated to "3HBzO") represented by the Chemical Formula [9]:

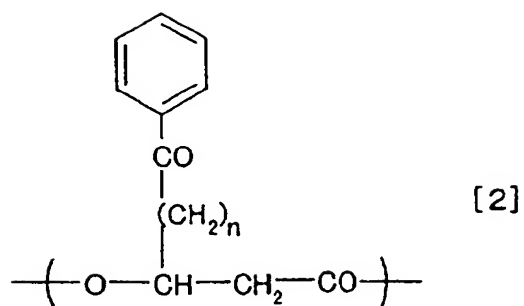


45 respectively. Further, it was found that the present PHAs can be biosynthesized by culturing these microorganisms under coexisting of BzBA, BzVA, BzHxA, BzHpA or BzOA with saccharides and that the present PHAs obtained had relatively high purity, thereby leading to complete the present invention.

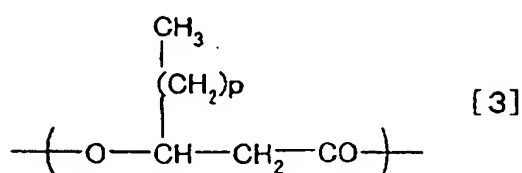
[0049] In other words, the present invention relates to polyhydroxyalkanoates having a composition of monomer units represented by the following Formula [1]:



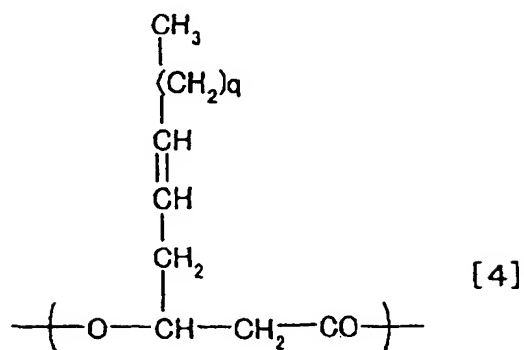
55 wherein A is at least one or more monomer units represented by the following Chemical Formula [2], B is at least or more selected from monomer units represented by the following Chemical Formula [3] or [4], and x is not less than 0.01 to less than 1.



15 wherein n is any integer of 1 to 8.

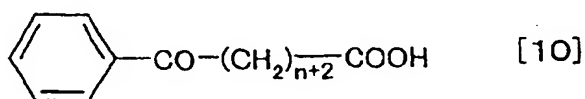


25 wherein p is any integer of 0 to 10.

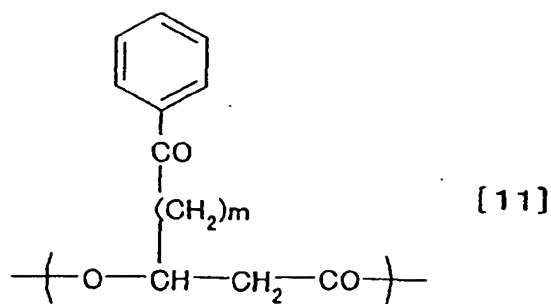


40 wherein q is 3 or 5.

**[0050]** In addition, the present invention relates to a method for producing polyalkanoates, comprising the step of culturing microorganisms in the medium containing benzoylalkanoic acids represented by the Chemical Formula [10]:



50 wherein n is any integer of 1 to 8,  
and producing, by the microorganisms, the polyalkanoates having the corresponding monomer units represented by the Chemical Formula [11]:



15 wherein m is at least one or more selected from the group consisting of n, n-2, n-4 and n-6, and is an integer not smaller than 1.

[0051] Herein, according to the present methods, saccharide compounds, for example, glucose, fructose, mannose and the like are used as substrates for growth of microorganisms so that monomer units derived from saccharides such as glucose are not contained at all or contained little in PHAs produced. From such a viewpoint, the present method differs fundamentally from the conventional methods for producing microbial PHAs using saccharides such as glucose.

[0052] According to the present invention, it is preferable to further have a step of isolating the PHAs produced by microorganisms.

[0053] According to the present invention, novel polyhydroxyalkanoates comprising 3-hydroxybenzoylalkanoic acids as monomer units and a method for producing the polyhydroxyalkanoates using microorganisms are provided. The polyhydroxyalkanoates useful as functional polymers can be efficiently produced whereby they can be expected to be applicable to each field such as device materials and medical materials.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0054]

FIG. 1 is a graphical representation showing the  $^1\text{H}$ -NMR spectrum of a polymer using BzBA as a starting material in Example 1;

FIG. 2 is a graphical representation showing the  $^1\text{H}$ -NMR spectrum of a polymer using BzVA as a starting material in Example 3;

FIG. 3 is a graphical representation showing the  $^{13}\text{C}$ -NMR spectrum of a polymer using BzVA as a starting material in Example 3;

FIG. 4 is a graphical representation showing the  $^1\text{H}$ -NMR spectrum of a polymer using BzHxA as a starting material in Example 1;

FIG. 5 is a graphical representation showing the  $^1\text{H}$ -NMR spectrum of a polymer using BzHpA as a starting material in Example 9; and

FIG. 6 is a graphical representation showing the  $^1\text{H}$ -NMR spectrum of a polymer using BzOA as a starting material in Example 12.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0055] PHAs of the present invention are isotactic polymers generally composed of only the R-form. <Organic Acids Associated with Saccharides and the TCA Cycle: Difference from Conventional Arts>

[0056] One of the methods for producing PHAs according to the present invention is characterized in that the contents of the objective monomer units are extremely increased or only the objective monomer units are obtained in the PHAs produced and accumulated by the microorganisms by adding only saccharides or organic acids associated with the TCA cycle as carbon sources other than the alkanoates, in addition to alkanoates for introduction of the desired monomer units, into the medium when culturing microorganisms. This accelerating effect of prioritizing the specified monomer units is obtained by adding only saccharides or organic acids associated with the TCA cycle as carbon sources other than the alkanoates into the medium.

[0057] In other words, the inventors have completed the present invention by obtaining the findings that the objective PHAs are obtained in much superior yields and purity, compared with the conventional methods using mol-alkanoates such as nonanoic acid and octanoic acid as coexisting substrates, when culturing saccharides or organic acids asso-

ciated with the TCA cycle as coexisting substrates together with alkanoates for introduction of the desired monomer units, and that such effect is obtained by the culturing method capable of generating acetyl-CoA, which is a carbon source and an energy source of microorganisms, by the method not depending on the  $\beta$ -oxidation.

**[0058]** According to the present invention, saccharide compounds, for example, glucose, fructose, mannose and the like are used as substrates for growth of microorganisms so that PHAs produced are composed of alkanoates for introduction of the desired monomer units coexisting with saccharides and the monomer units derived from the saccharides such as glucose are not contained at all or contained extremely little in them. From such a viewpoint, the present methods differ fundamentally in both the constitution and effect from the conventional methods for producing microbial PHAs using saccharides themselves such as glucose as starting substrates for introducing monomer units into PHAs.

**[0059]** The PHAs, production method and microorganisms of the present invention will be described in details below.

#### <Supplying System of PHA Monomer Units>

**[0060]** First, the "fatty acid synthesis pathway", which is one of systems supplying mcl-3HA monomer units being mixed into the objective PHAs will be described in details.

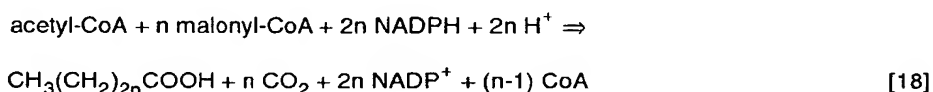
**[0061]** In the case where saccharides such as glucose are substrates, alkanoates necessary for cellular components are biosynthesized through the "fatty acid synthesis pathway" in which acetyl-CoA produced from saccharides through the "glycolytic pathway" is a starting substance. The fatty acid synthesis includes the de novo synthetic pathway and the carbon-chain elongation pathway, which will be described below.

#### 1) De Novo Synthetic Pathway

**[0062]** This pathway is catalyzed by two enzymes which are acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthase (EC 2.3.1.85). The acetyl-CoA carboxylase is an enzyme interposing biotin, ultimately catalyzing the following reaction to produce malonyl-CoA from acetyl-CoA. The reaction is represented by the following Scheme [17].

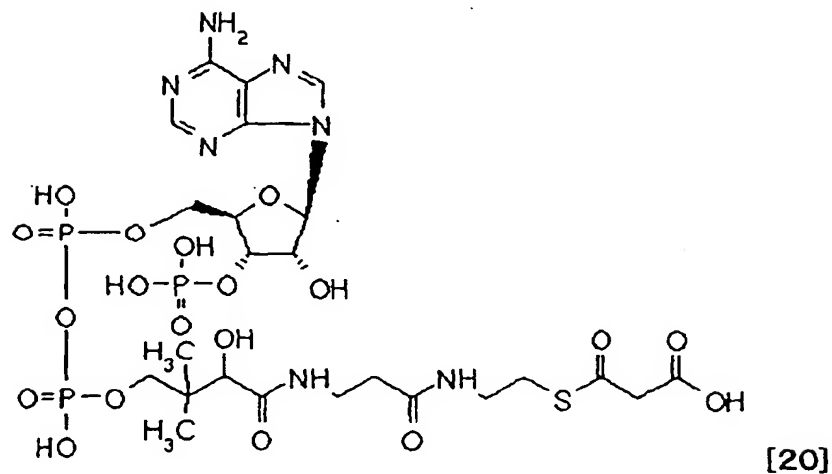
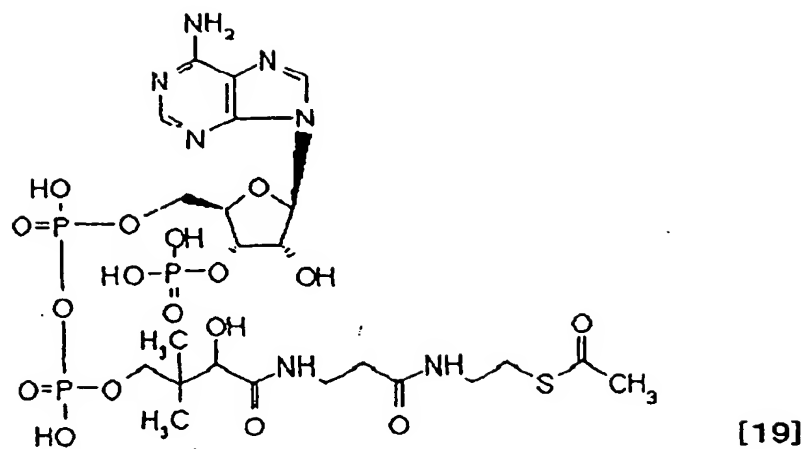


**[0063]** The fatty acid synthase is an enzyme catalyzing the reaction cycle of transfer-condensation-reduction-dehydration-reduction. The entire reactions are represented by the following Reaction Scheme [18].

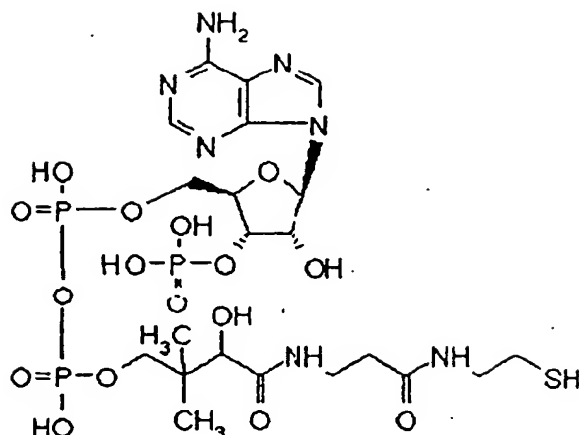


**[0064]** Herein, the reaction products may be free acids, CoA-derivatives or ACP-derivatives, depending on the type of enzymes.

**[0065]** Herein, the acetyl-CoA and malonyl-CoA are represented by the following Chemical Formulas [19] and [20].

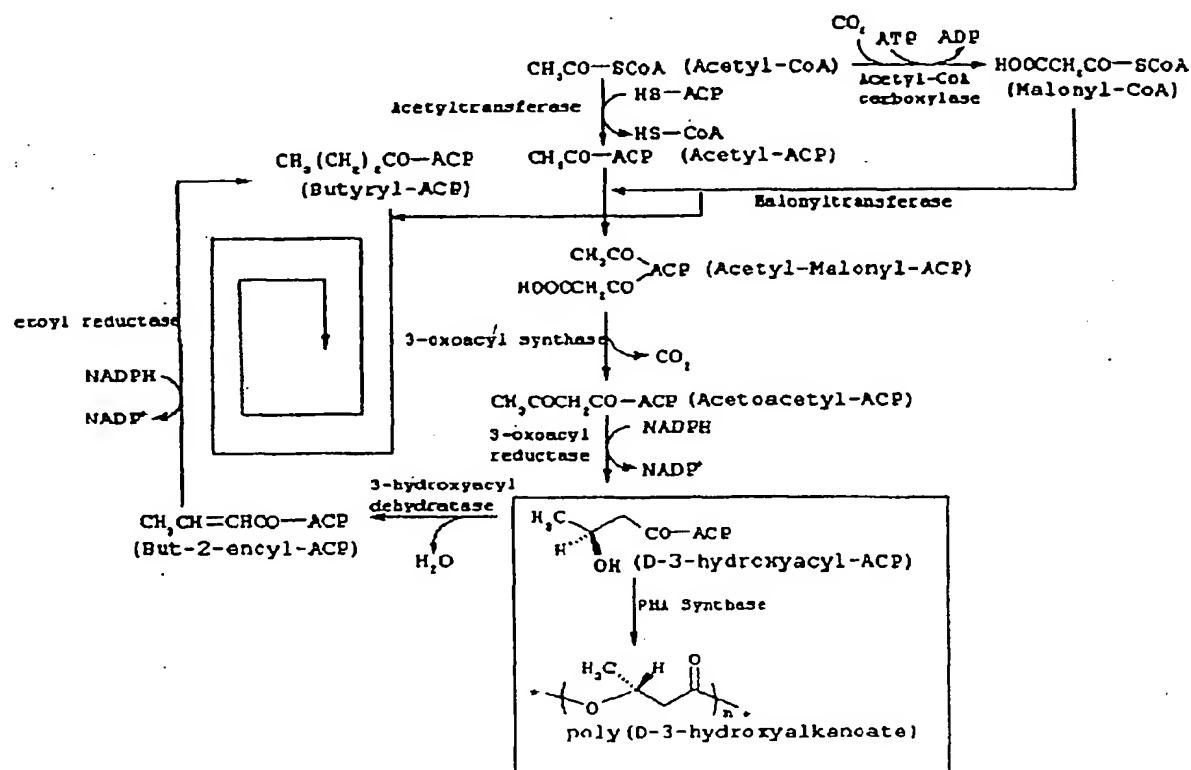


40 [0066] In addition, CoA is abbreviation of co-enzyme A represented by the following Chemical Formula [21].



[21]

[0067] In this reaction pathway, "D-3-hydroxyacyl-ACP" which is to be the monomer substrate for the PHA biosynthesis is supplied as an intermediate through the route described below. Additionally, routes as shown in the following reaction schemes are extended finally to palmitic acid with repeated addition of two carbons. Therefore, as the monomer substrate for the PHA biosynthesis are provided seven "D-3-hydroxyacyl-ACPs" having even numbers of carbons, from "D-3-hydroxybutyryl-ACP" to "D-3-hydroxypalmityl-ACP".

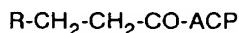
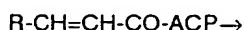
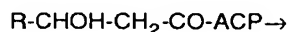
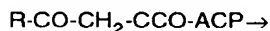


## 2) Carbon-Chain Elongation Pathway

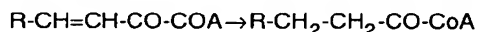
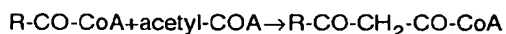
[0068] This pathway is broadly divided into two pathways:

in one of which, malonyl-ACP is added to acyl-ACP to ultimately convert them into acyl-ACP having the carbon chain extended with two carbons (and CO<sub>2</sub>) (referred to as "Pathway A"), and in another, acetyl-CoA is added to acyl-CoA to ultimately convert them into acyl-CoA having the carbon chain extended with two carbons (referred to as "Pathway B"). Each pathway will be described below.

#### Pathway A



#### Pathway B



**[0069]** In both Pathways A and B; it is thought that "D-3-hydroxyacyl-CoA" or "D-3-hydroxyacyl-ACP" is yielded as an intermediate, and "D-3-hydroxyacyl-CoA" is utilized as the monomer substrate for the PHA synthesis as it is, while "D-3-hydroxyacyl-ACP" is utilized as the monomer substrate for the PHA synthesis after being converted to "D-3-hydroxyacyl-CoA" by ACP-CoA transferase.

**[0070]** In the case where saccharides such as glucose and the like are used as a substrate, it is thought that an mcl-3HA monomer unit is formed via the "glycolytic pathway" and the "fatty acid synthesis pathway" within the microbial cells, as described above. In the case where organic acids involved in the TCA cycle are used as a substrate, acetyl-CoA is yielded directly from pyruvic acid by pyruvate dehydrogenase. Organic acids on the TCA cycle, for example, malic acid yields pyruvic acid by malate dehydrogenase, further, acetyl-CoA is yielded from the above-described reaction. Oxaloacetic acid yields phosphoenolpyruvic acid by phosphoenolpyruvate kinase, phosphoenolpyruvic acid yields pyruvic acid catalyzed by pyruvate kinase, further acetyl-CoA is generated from the above-described reaction. It is considered that Acetyl-CoA generated by these reactions yields the mcl-3HA monomer unit via the "fatty acid synthesis pathway".

**[0071]** It is considered that mcl-alkanoates, e.g. octanoic acid or nonanoic acid, or alkanoates which are added with a functional group other than the straight-chained aliphatic alkyl group at the terminal, e.g., 5-phenylvaleric acid, 5-(4-fluorophenyl)valeric acid, 6-phenylhexanoic acid, 4-phenoxybutyric acid or 4-cyclohexylbutyric acid are converted to CoA derivatives by CoA ligase (EC 6. 2. 1. 3, etc.), and converted to "D-3-hydroxyacyl-CoA" which becomes directly a monomer substrate of the PHA biosynthesis by the enzyme group functioning in the  $\beta$ -oxidation system.

**[0072]** In other words, it means that the mcl-3HA monomer unit formed from saccharides or organic acids associated with the TCA cycle is formed through extremely multistage-enzymatic reaction (i.e. indirectly), while the mcl-3HA monomer units are formed very directly from the mcl-alkanoates.

**[0073]** Herein, generation of acetyl-CoA carrying out growth of microorganisms will be described. In a method of coexisting with the mcl-alkanoates in addition to alkanoates for introduction of the objective monomer units, acetyl-CoA is generated through the  $\beta$ -oxidation system of these alkanoates. Comparing with alkanoates having a bulky substituent (alkanoates having substituents such as a phenyl group, phenoxy group or cyclohexyl group), generally the mcl-alkanoates is presumably excellent in the substrate affinity with the enzyme group of the  $\beta$ -oxidation system, so that acetyl-CoA is effectively generated by coexistence with the mcl-alkanoates. Therefore, it is advantageous for growth of microorganisms to use acetyl-CoA as both an energy source and a carbon source.

**[0074]** However, since the mcl-alkanoates via the  $\beta$ -oxidation system are converted directly into monomer units of

PHAs, it is a significant problem that a large amount of the mcl-3HA monomer units are mixed in addition to the objective monomer units.

[0075] In order to solve this problem, it is desirable to select the substrates other than the mcl-alkanoates capable of effectively supplying acetyl-CoA or an energy source and a carbon source, and to use the method of coexisting with the objective alkanoates. As described previously, although acetyl-CoA can be converted into monomer units of PHAs through the fatty acid synthesis pathway, it is necessary to pass through more multistage reactions as compared to the mcl-alkanoates, and it is indirectly yielded. By suitably selecting culture conditions such as concentration of substrates capable of generating acetyl-CoA, it is possible to carry out the production method in which the mcl-3HAs are not substantially mixed or mixed little.

[0076] The production method to be widely used comprises the first step of culturing only growth of microorganisms, and the second step of adding only the objective alkanoate as a carbon source into the medium. Herein, since acyl-CoA ligase which is a starting enzyme for converting the present alkanoate into acyl-CoA needs ATP, according to the inventors' study, the results that the production method of coexisting with substrates usable for microorganisms as an energy source also at the second step was more effective were obtained, thereby leading to complete the present invention.

[0077] Microorganisms, culture steps and the like utilized in the present invention will be described below.

(Microorganisms)

[0078] For microorganisms used in the present invention, if BzBA, BzVA, BzHxA, BzHpA or BzOA can be used as a starting material to produce the corresponding PHAs comprising the above-described 3HBzB, 3HBzV, 3HBzHx, 3HBzHp or 3HBzO as a monomer unit, respectively, any microorganisms may be used. Further, within the scope attainable for the purposes of the present invention, plural microorganisms may be mixed and used if necessary.

[0079] The present inventors performed screening of microorganisms capable of producing the corresponding PHA comprising the above-described 3HBzB, 3HBzV, 3HBzHx, 3HBzHp or 3HBzO as a monomer unit by using BzBA, BzVA, BzHxA, BzHpA or BzOA as a substrate, respectively, and accumulating them in the cells. As a result, the present inventors have found that microorganisms isolated from soil which have producibility of PHAs and the desired ability are *Pseudomonas cichorii* strain H45, *Pseudomonas cichorii* strain YN2, *Pseudomonas jessenii* strain P161 and the like. Herein, strain H45 as deposition No. "FERM BP-7374", strain YN2 as deposition No. "FERM BP-7375" and strain P161 as deposition No. "FERM BP-7376" have been deposited each in Deposition Center of Patent Microorganisms, Research Institute of Biotechnology and Industry, the Agency of Industrial Science of Technology, the Ministry of Economy and Industry, and are the microorganisms described in Japanese Patent Application No. 11-371863. In addition, the international deposition for these microorganisms has been carried out according to the Budapest Treaty. The international requisition and deposition Nos. for these microorganisms are as follows, strain H45: "FERM BP-7374", strain YN2: "FERM BP-7375" and strain P161: "FERM BP-7376".

[0080] The bacteriological properties of the above-described strains H45, YN2 and P161 will be enumerated as follows. For strain P161, the basic sequence of 16SrRNA will be shown in sequence No. 1.

<Bacteriological Properties of Strain H45>

[0081]

#### (1) Morphological Properties

Shape and size of cells: rod, 0.8  $\mu\text{m}$   $\times$  1.0 to 1.2  $\mu\text{m}$   
 Polymorphism of cells: negative  
 Mobility: motile  
 Sporulation: negative  
 Gram staining: negative  
 Colony shape: circular; entire, smooth margin; low  
 convex, smooth surface; glossy; cream-colored

#### (2) Physiological Properties

Catalase: positive  
 Oxidase: positive  
 O/F test: oxidative  
 Nitrate reduction: negative



EP 1 130 042 A2

Indole production: negative  
Acid production from glucose: negative  
Arginine dihydrolase: negative  
Urease: negative  
Esculin hydrolysis: negative  
Gelatin hydrolysis: negative  
 $\beta$ -Galactosidase: negative  
Fluorescent pigment production on King's B agar: positive  
Growth under 4% NaCl: negative  
Poly- $\beta$ -hydroxybutyrate accumulation: negative

(3) Substrate Assimilation

Glucose: positive  
L-Arabinose: negative  
D-Mannose: positive  
D-Mannitol: positive  
N-Acetyl-D-glucosamine: positive  
Maltose: negative  
Potassium gluconate: positive  
n-Capric acid: positive  
Adipic acid: negative  
dl-Malic acid: positive  
Sodium citrate: positive  
Phenyl acetate: positive

<Bacteriological Properties of Strain YN2>

[0082]

(1) Morphological Properties

Shape and size of cells: rod,  $0.8\text{ }\mu\text{m} \times 1.5\text{ to }2.0\text{ }\mu\text{m}$  Polymorphism of cells: negative  
Mobility: motile  
Sporulation: negative  
Gram staining: negative  
Colony shape: circular; entire, smooth margin; low convex, smooth surface; glossy; translucent

(2) Physiological Properties

Catalase: positive  
Oxidase: positive  
O/F test: oxidative  
Nitrate reduction: negative  
Indole production: positive  
Acid production from glucose: negative  
Arginine dihydrolase: negative  
Urease: negative  
Esculin hydrolysis: negative  
Gelatin hydrolysis: negative  
 $\beta$ -Galactosidase: negative  
Fluorescent pigment production on King's B agar: positive  
Growth under 4% NaCl: positive (weak growth)  
Poly- $\beta$ -hydroxybutyrate accumulation: negative Tween 80 hydrolysis: positive

(3) Substrate Assimilation

Glucose: positive

L-Arabinose: positive  
D-Mannose: negative  
D-Mannitol: negative  
N-Acetyl-D-glucosamine: negative  
5 Maltose: negative  
Potassium gluconate: positive  
n-Capric acid: positive  
Adipic acid: negative  
10 dl-Malic acid: positive  
Sodium citrate: positive  
Phenyl acetate: positive

<Bacteriological Properties of Strain P161>

15 [0083]

(1) Morphological Properties

20 Shape and size of cells: sphere,  $\phi 0.6 \mu\text{m}$   
rod,  $0.8 \mu\text{m} \times 1.5$  to  $2.0 \mu\text{m}$   
Polymorphism of cells: positive (extended type) Mobility: motile  
Sporulation: negative  
Gram staining: negative  
25 Colony shape: circular; entire, smooth margin; low  
convex, smooth surface; glossy; pale yellow

(2) Physiological Properties

30 Catalase: positive  
Oxidase: positive  
O/F test: oxidative  
Nitrate reduction: positive  
Indole production: negative  
Acid production from glucose: negative  
35 Arginine dihydrolase: positive  
Urease: negative  
Esculin hydrolysis: negative  
Gelatin hydrolysis: negative  
 $\beta$ -Galactosidase: negative  
40 Fluorescent pigment production on King's B agar: positive

(3) Substrate Assimilation

45 Glucose: positive  
L-Arabinose: positive  
D-Mannose: positive  
D-Mannitol: positive  
N-Acetyl-D-glucosamine: positive  
Maltose: negative  
50 Potassium gluconate: positive  
n-Capric acid: positive  
Adipic acid: negative  
dl-Malic acid: positive  
Sodium citrate: positive  
55 Phenyl acetate: positive

(Culture Step)

<General Culture>

**[0084]** The objective PHAs can be produced by culturing these microorganisms in the medium containing alkanoates for introduction of the desired monomer units and substrates for growth according to the present invention. Such PHAs are generally composed of only the R-form and are isotactic polymers.

**[0085]** In usual culture of microorganisms used for the production methods of PHAs according to the present invention, for example, preparation of stock cell strains, the cell count necessary for production of PHAs and growth for holding the active state and the like, the media containing requisite components for growth of microorganisms to be used are suitably selected and used. For example, any kinds of media such as general natural media (nutrient broth, yeast extract, etc.) and synthetic media added with nutrients may be used as long as they do not have a bad influence on growth and survival of microorganisms.

**[0086]** Any of culture methods using the culture such as the liquid culture, solid culture and the like can be used as long as the microorganisms can grow and produce the PHAs. Further, it may use any types: batch culture, fed batch culture, semicontinuous culture, continuous culture and the like. As forms of the liquid batch culture, methods for supplying oxygen include the shaking one using a shaking flask and the spinner aeration one using a jar fermenter. The multistage method in which these processes are connected to plural steps may be also adopted.

**[0087]** In the case where PHAs comprising 3HBzB, 3HBzV, 3HBzHx, 3HBzHp or 3HBzO as a monomer unit are produced using the above-described microorganisms, the inorganic media and the like may be used which contain at least the corresponding BzBA, BzVA, BzHxA, BzHpA or BzOA as a starting material for the PHA production, respectively, and carbon sources for growth of microorganisms. For the carbon sources for growth, nutrients such as yeast extract, polypeptone and meat extract can be used, further, they includes: saccharides, for example, aldoses such as glyceraldehyde, erythrulose, arabinose, xylose, glucose, galactose, mannose and fructose, alditols such as glycerol, erythritol and xylitol, aldonic acids such as gluconic acid, uronic acids such as glucuronic acid and galacturonic acid, disaccharides such as maltose, sucrose and lactose, further organic acids or their salts such as pyruvic acid, malic acid, citric acid and succinic acid which are formed as intermediates in the TCA cycle, amino acids or their salts such as glutamic acid and the like, and if the compounds can yield acetyl-CoA without passing through the  $\beta$ -oxidation cycle, any of them can be used and suitably selected as substrates useful for cell strains to be used. Also, if the combination has little mixture of mcl-3HA, it is possible to select and use plural compounds. Among them, it is preferable to use particularly saccharides, more preferably at least one selected from the group consisting of glucose, fructose and mannose. As a method of producing and accumulating PHAs by microorganisms, there is a method in which once they are grown sufficiently, then the cells are transferred to the medium in which a nitrogen source such as ammonium chloride is limited, the compounds to become substrates of the desired units are added to the medium and the cells are further cultured in this condition, whereby the producibility is improved in some cases. Specifically, it includes adoption of the multistage method in which the above-described processes are connected to plural steps. For example, there is a culture method in which after being cultured up to the point of steady state from the latter term of logarithmic growth in the inorganic media and the like containing about 0.05% to 5.0% of D-glucose and about 0.01% to 1.0% of BzBa, BzVA, BzHxA, BzHpA or BzOA, the cells are recovered with centrifugal separation and the like, and they are, further, cultured in the inorganic media containing about 0.01% to 1.0% of BzBA, BzVA, BzHxA, BzHpA or BzOA where nitrogen sources are limited to or substantially not present.

**[0088]** As the inorganic media to be used for the above-described culture method, any of them may be used provided that they contains the components such as phosphorus sources (e.g., phosphate, etc.) and nitrogen sources (e.g., ammonium salts, nitrate, etc.) by which microorganisms can grow, the inorganic salt media can include, for example, the MSB medium, E medium (J. Biol. Chem., 218, 97-106 (1956)), M9 medium or the like.

**[0089]** Herein, composition of the M9 medium used in Examples of the present invention is as follows.

Na<sub>2</sub>HPO<sub>4</sub>: 6.2 g  
KH<sub>2</sub>PO<sub>4</sub>: 3.0g  
NaCl: 0.5 g  
NH<sub>4</sub>Cl: 1.0 g  
(in 1 liter medium, pH 7.0)

**[0090]** For the better growth and production of PHAs, about 0.3% (v/v) solution of minor components, as shown below, preferably is added to the above-described inorganic salt medium.

## EP 1 130 042 A2

[Minor Component Solution]

[0091]

5        Nitrilotriacetic acid: 1.5 ;  $\text{MgSO}_4$ : 3.0 ;  
          $\text{MnSO}_4$ : 0.5 ;  $\text{NaCl}$ : 1.0 ;  $\text{FeSO}_4$ : 0.1 ;  
          $\text{CaCl}_2$ : 0.1 ;  $\text{CoCl}_2$  ;  $\text{ZnSO}_4$ : 0.1 ;  
          $\text{CuSO}_4$ : 0.1 ;  $\text{AlK}(\text{SO}_4)_2$ : 0.1 ;  
10         $\text{H}_3\text{BO}_3$ : 0.1 ;  $\text{Na}_2\text{MoO}_4$ : 0.1 ;  $\text{NiCl}_2$ : 0.1  
         (in 1 liter)

[0092] For the culture temperature, it may be a temperature enabling good growth of the above-described cell strains, for example, 14 to 40°C, preferably about 20 to 35°C are appropriate.

15        [0093] As a specific example, after being cultured in the inorganic media and the like containing about 0.05% to 5.0% of D-glucose and about 0.01% to 1.0% of BzBA, BzVA, BzHxA, BzHpA or BzOA followed by recovering the cells at the point from the latter term of logarithmic growth to the steady state, the desired PHAs can be extracted in which the unobjective monomer units are less mixed or are not present at all. Such PHAs are generally composed of only the R-form and are isotactic polymers.

20        [0094] The same amount of yeast extract in place of D-glucose may be given. In addition, it may use polypeptone, organic acids (e.g. lactic acid, pyruvic acid, citric acid, succinic acid, fumaric acid, malic acid, etc. and their salts) associated with the TCA cycle and their combination.

<Recovery of PHAs>

25        [0095] For obtaining PHAs from the culture solutions according to the present invention, the methods to be usually conducted can be applied. In the case where the PHAs are discharged in the culture solution, the methods for extraction from the culture solution and for their purification are used, and in the case where they are accumulated in the cells, the methods for extraction from the cells and their purification are used. For example, the most simple method for recovery of PHAs from the cultured cells of microorganisms is carried out by extraction with organic solvents such as chloroform which is usually conducted, while acetone other than chloroform is used sometimes. Also in the circum-  
30        stance unlikely to use organic solvents, a method can be used in which PHAs are recovered after removing the cell components other than PHAs by treating with surfactants such as SDS, enzymes such as lysozyme and chemical agents such as EDTA, sodium hypochlorite, ammonia and the like.

35        [0096] Culturing the microorganisms of the present invention, producing PHAs by the microorganisms of the present invention and accumulating them in the cells and recovering PHAs from the cells according to the present invention are not limited to the above described methods.

[0097] Examples will be shown below. Herein, the following "%" is based on the weight unless otherwise marked.

[Example 1]

40        [0098] *Pseudomonas cichorii* strain YN2 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzBA followed by being cultured with shaking at 30°C at 125 strokes/min. After 48 hr, the cells were recovered by centrifugal separation, then resuspended into 200 ml of the M 9 medium containing 0.5% D-glucose and 0.1% BzBA without containing a nitrogen source ( $\text{NH}_4\text{Cl}$ ), followed by being cultured with shaking at 30°C at 125 strokes/min. After  
45        42 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and lyophilized.

[0099] This lyophilized pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45  $\mu\text{m}$ , it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

50        [0100] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 3, it was confirmed that the present PHA is PHA containing 3HBzB as monomer units.

Table 3

Production of PHA by <i>Pseudomonas cichorii</i> strain YN2	
Dry Weight of Cell	310 mg/L

# EP 1 130 042 A2

Table 3 (continued)

Production of PHA by <i>Pseudomonas cichorii</i> strain YN2	
Dry weight of Polymer	10 mg/L
Monomer Unit Composition (peak area ratio)	
3-hydroxyhexanoic acid	3%
3-hydroxyoctanoic acid	26%
3-hydroxydecanoic acid	32%
3-hydroxydodecanoic acid	14%
3-hydroxydodecenoic acid	17%
3-hydroxy-4-benzoylbutyric acid	8%

[0101] This PHA was analyzed in the following measurement conditions using the NMR spectrometer (FT-NMR: Bruker DPX400).

<Measurement Conditions>

[0102]

Measuring nuclide:  $^1\text{H}$   
 Solvent used:  $\text{CDCl}_3$   
 (TMS/ $\text{CDCl}_3$  sealed with a capillary was used as a reference)  
 Resonance frequency:  $^1\text{H} = 400 \text{ MHz}$   
 The  $^1\text{H}$ -NMR spectrum is shown in FIG. 1.

[0103] From the  $^1\text{H}$ -NMR spectrum shown in FIG. 1, it was found that the PHA having monomer units represented by the above-described Chemical Formula [2] was obtained.

[0104] Further, the molecular weight of the PHA was evaluated by Gel Permeation Chromatography (GPC; Tosoh HLC-8020, column; Polymer Laboratory PL gel MIXED-C (5  $\mu\text{m}$ ), solvent; chloroform, converted into polystyrene), thereby obtaining  $M_n = 18,000$  and  $M_w = 42,000$ .

[Example 2]

[0105] *Pseudomonas jessenii* strain P161 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzBA followed by being cultured with shaking at 30°C at 125 strokes/min. After 48 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzBA without containing a nitrogen source ( $\text{NH}_4\text{Cl}$ ), followed by being cultured with shaking at 30°C at 125 strokes/min. After 42 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and lyophilized.

[0106] This lyophilized pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and the PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45  $\mu\text{m}$ , it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

[0107] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 4, it was confirmed that the present PHA is PHA containing 3HBzB as monomer units.

Table 4

Production of PHA by <i>Pseudomonas jessenii</i> strain P161	
Dry Weight of Cell	310 mg/L
Dry weight of Polymer	25 mg/L
Monomer Unit Composition (peak area ratio)	
3-hydroxyhexanoic acid	3%
3-hydroxyoctanoic acid	32%

# EP 1 130 042 A2

Table 4 (continued)

Monomer Unit Composition (peak area ratio)	
3-hydroxynonanoic acid	2%
3-hydroxydecanoic acid	32%
3-hydroxydodecanoic acid	7%
3-hydroxydodecenoic acid	24%
3-hydroxy-4-benzoylbutyric acid	1%

[Example 3]

**[0108]** *Pseudomonas cichorii* strain YN2 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzVA followed by being cultured with shaking at 30°C at 125 strokes/min. After 48 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzVA without containing a nitrogen source (NH<sub>4</sub>Cl), followed by being cultured with shaking at 30°C at 125 strokes/min. After 42 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and lyophilized.

**[0109]** This lyophilized pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45 µm, it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

**[0110]** After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 5, it was confirmed that the present PHA is PHA containing 3HBzV as monomer units.

Table 5

Production of PHA by <i>Pseudomonas cichorii</i> strain YN2	
Dry Weight of Cell	890 mg/L
Dry weight of Polymer	300 mg/L
Monomer Unit Composition (peak area ratio)	
3-hydroxyoctanoic acid	3%
3-hydroxydecanoic acid	9%
3-hydroxydodecanoic acid	3%
3-hydroxydodecenoic acid	5%
3-hydroxy-5-benzoylvaleric acid	80%

**[0111]** This PHA was analyzed in the following measurement conditions using the NMR spectrometer (FT-NMR: Bruker DPX400).

<Measurement Conditions>

**[0112]**

Measuring nuclide: <sup>1</sup>H, <sup>13</sup>C

Solvent used: CDCl<sub>3</sub>

(TMS/CDCl<sub>3</sub> sealed with a capillary was used as a reference)

Resonance frequency: <sup>1</sup>H = 400 MHz, <sup>13</sup>C = 100 MHz

The <sup>1</sup>H- and <sup>13</sup>C-NMR spectra are shown in FIGS. 2 and 3.

**[0113]** From the <sup>1</sup>H- and <sup>13</sup>C-NMR spectra shown in FIGS. 2 and 3, it was found that the PHA having monomer units represented by the above-described Chemical Formula [3] was obtained. The assignment results (see Chemical Formula [22]) are shown in Table 6.

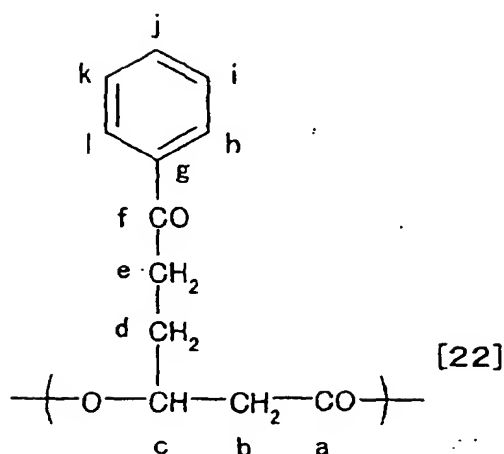


Table 6

Assignment of $^1\text{H}$ and $^{13}\text{C}$ -NMR Spectrum				
$^1\text{H}$				$^{13}\text{C}$
Location	Chemical Shift/ppm	Integral Value	Type	Chemical Shift/ppm
a	-	-	-	169.3
b	2.56	2	m	39.2
c	5.26	1	m	70.2
d	2.04	2	m	28.0
e	3	2	m	34.0
f	-	-	-	198.6
g	-	-	-	136.5
h, l	7.89	2	d	127.9
i, k	7.36	2	m	128.5
j	7.46	1	t	133.0
d: doublet, t: triplet, m: multiplet				

[0114] Further, the molecular weight of the PHA was evaluated by Gel Permeation Chromatography (GPC; Tosoh HLC-8020, column; Polymer Laboratory PL gel MIXED-C (5  $\mu\text{m}$ ), solvent; chloroform, converted into polystyrene), thereby obtaining  $M_n = 330,000$  and  $M_w = 1,300,000$ .

#### [Example 4]

[0115] *Pseudomonas cichorii* strain H45 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzVA followed by being cultured with shaking at 30°C at 125 strokes/min. After 48 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzVA without containing a nitrogen source ( $\text{NH}_4\text{Cl}$ ), followed by being cultured with shaking at 30°C at 125 strokes/min. After 42 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and lyophilized.

[0116] This lyophilized pellet was suspended into 20 mL chloroform, stirred at 60°C for 20 hr and the PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45  $\mu\text{m}$ , it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

[0117] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 7, it was confirmed that the present PHA is PHA containing 3HBzV as monomer units.

Table 7

Production of PHA by <i>Pseudomonas cichorii</i> strain H45	
Dry Weight of Cell	770 mg/L
Dry weight of Polymer	270 mg/L
Monomer Unit Composition (peak area ratio)	
3-hydroxyoctanoic acid	5%
3-hydroxydecanoic acid	9%
3-hydroxydodecanoic acid	3%
3-hydroxydodecenoic acid	3%
3-hydroxy-5-benzoylvaleric acid	80%

[Example 5]

**[0118]** *Pseudomonas jessenii* strain P161 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzVA followed by being cultured with shaking at 30°C at 125 strokes/min. After 46 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzVA without containing a nitrogen source (NH<sub>4</sub>Cl), followed by being cultured with shaking at 30°C at 125 strokes/min. After 41 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and lyophilized.

**[0119]** This lyophilized pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45 µm, it was concentrated with a rotary evaporator, and the concentrate was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

**[0120]** After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 8, it was confirmed that the present PHA is PHA containing 3HBzV as monomer units.

Table 8

Production of PHA by <i>Pseudomonas jessenii</i> strain P161	
Dry Weight of Cell	980 mg/L
Dry weight of Polymer	420 mg/L
Monomer Unit Composition (peak area ratio)	
3-hydroxybutyric acid	1%
3-hydroxyoctanoic acid	7%
3-hydroxydecanoic acid	13%
3-hydroxydodecanoic acid	3%
3-hydroxydodecenoic acid	4%
3-hydroxy-5-benzoylvaleric acid	72%

[Example 6]

**[0121]** *Pseudomonas cichorii* strain YN2 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHxA followed by being cultured with shaking at 30°C at 125 strokes/min. After 46 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHxA without containing a nitrogen source (NH<sub>4</sub>Cl), followed by being cultured with shaking at 30°C at 125 strokes/min. After 47 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and vacuum-dried.

**[0122]** This vacuum-dried pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45 µm, it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

**[0123]** The obtained PHA was analyzed in the following measurement conditions using the NMR spectrometer (FT-



NMR: Bruker DPX400).

<Measurement Conditions>

[0124]

Measuring nuclide:  $^1\text{H}$

Solvent used:  $\text{CDCl}_3$

(TMS/ $\text{CDCl}_3$  sealed with a capillary was used as a reference)

Resonance frequency:  $^1\text{H} = 400 \text{ MHz}$

The  $^1\text{H}$ -NMR spectra are shown in FIG. 4.

[0125] From the  $^1\text{H}$ -NMR spectra shown in FIG. 4, it was found that the PHA having monomer units represented by the above-described Chemical Formula [4] was obtained.

[0126] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. The results are shown in Table 9.

Table 9

Production of Polyhydroxyalkanoates by Strain YN2	
Dry Weight of Cell (mg/L)	710
Weight of Polymer (mg/L)	180
Monomer Unit Composition (peak area ratio)	
3-hydroxyhexanoic acid	1.0%
3-hydroxyoctanoic acid	8.4%
3-hydroxydecanoic acid	8.9%
3-hydroxydodecanoic acid	4.4%
3-hydroxydodecenoic acid	7.2%
3-hydroxytetradecanoic acid	0.3%
3-hydroxy-6-benzoylhexanoic acid	69.8%

[0127] From the above results, it was confirmed that the present PHA is PHA containing 3HBzHx as monomer units.

[0128] Further, the molecular weight of the PHAs was evaluated by Gel Permeation Chromatography (GPC; Tosoh HLC-8020, column; Polymer Laboratory PL gel MIXED-C (5  $\mu\text{m}$ ), solvent; chloroform, converted into polystyrene), thereby obtaining  $M_n = 24,000$  and  $M_w = 62,000$ .

[Example 7]

[0129] *Pseudomonas cichorii* strain H45 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHxA followed by being cultured with shaking at 30°C at 125 strokes/min. After 46 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHxA without containing a nitrogen source ( $\text{NH}_4\text{Cl}$ ), followed by being cultured with shaking at 30°C at 125 strokes/min. After 47 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and vacuum-dried.

[0130] This vacuum-dried pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and the PHAs were extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45  $\mu\text{m}$ , it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

[0131] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 10, it was confirmed that the present PHA is PHA containing 3HBzHx as monomer units.

# EP 1 130 042 A2

Table 10

Production of Polyhydroxyalkanoates by Strain H45	
Dry Weight of Cell (mg/L)	760
Weight of Polymer (mg/L)	140
Monomer Unit Composition (peak area ratio)	
3-hydroxyhexanoic acid	1.4%
3-hydroxyoctanoic acid	10.7%
3-hydroxynonanoic acid	0.1%
3-hydroxydecanoic acid	8.4%
3-hydroxydodecanoic acid	2.7%
3-hydroxydodecenoic acid	3.5%
3-hydroxy-6-benzoylhexanoic acid	73.2%

[Example 8]

**[0132]** *Pseudomonas jessenii* strain P161 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHxA followed by being cultured with shaking at 30°C at 125 strokes/min. After 46 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHxA without containing a nitrogen source (NH<sub>4</sub>Cl), followed by being cultured with shaking at 30°C at 125 strokes/min. After 47 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and lyophilized.

**[0133]** This lyophilized pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45 µm, it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

**[0134]** After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 11, it was confirmed that the present PHA is PHA containing 3HBzHx as monomer units.

Table 11

Production of Polyhydroxyalkanoates by Strain P161	
Dry Weight of Cell (mg/L)	540
Weight of Polymer (mg/L)	200
Monomer Unit Composition (peak area ratio)	
3-hydroxybutyric acid	0.2%
3-hydroxyhexanoic acid	2.2%
3-hydroxyoctanoic acid	21.1%
3-hydroxynonanoic acid	0.1%
3-hydroxydecanoic acid	15.9%
3-hydroxydodecanoic acid	2.9%
3-hydroxydodecenoic acid	6.0%
3-hydroxytetradecanoic acid	0.7%
3-hydroxy-6-benzoylhexanoic acid	50.9%

[Example 9]

**[0135]** *Pseudomonas cichorii* strain YN2 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHpA followed by being cultured with shaking at 30°C at 125 strokes/min. After 47 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHpA without containing a nitrogen source (NH<sub>4</sub>Cl), followed by being cultured with shaking at 30°C at 125

strokes/min. After 43 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and vacuum-dried.

[0136] This vacuum-dried pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45 µm, it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

[0137] The obtained PHA was analyzed in the following measurement conditions using the NMR spectrometer (FT-NMR: Bruker DPX400).

#### <Measurement Conditions>

#### [0138]

Measuring nuclide:  $^1\text{H}$

Solvent used:  $\text{CDCl}_3$

(TMS/ $\text{CDCl}_3$  sealed with a capillary was used as a reference)

Resonance frequency:  $^1\text{H}$  = 400 MHz

The  $^1\text{H}$ -NMR spectra are shown in FIG. 5.

[0139] From the  $^1\text{H}$ -NMR spectra shown in FIG. 5, it was found that the PHA having monomer units represented by the above described Chemical Formula [5] was obtained.

[0140] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. The results are shown in Table 12.

Table 12

Production of Polyhydroxyalkanoates by Strain YN2	
Dry Weight of Cell (mg/L)	1440
Weight of Polymer (mg/L)	590
Monomer Unit Composition (peak area ratio)	
3-hydroxybutyric acid	0.3%
3-hydroxyhexanoic acid	0.8%
3-hydroxyoctanoic acid	6.8%
3-hydroxydecanoic acid	14.3%
3-hydroxydodecanoic acid	4.6%
3-hydroxydodecenoic acid	8.2%
3-hydroxy-5-benzoylvaleric acid	30.8%
3-hydroxy-7-benzoylheptanoic acid	34.2%

[0141] From the above results, it was confirmed that the present PHA is PHA containing 3HBzHp as monomer units.

[0142] Further, the molecular weight of the PHAs was evaluated by Gel Permeation Chromatography (GPC; Tosoh HLC-8020, column; Polymer Laboratory PL gel MIXED-C (5 µm), solvent; chloroform, converted into polystyrene), thereby obtaining  $M_n$  = 23,000 and  $M_w$  = 53,000.

#### [Example 10]

[0143] *Pseudomonas cichorii* strain H45 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHpA followed by being cultured with shaking at 30°C at 125 strokes/min. After 47 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHpA without containing a nitrogen source ( $\text{NH}_4\text{Cl}$ ), followed by being cultured with shaking at 30°C at 125 strokes/min. After 43 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and vacuum-dried.

[0144] This vacuum-dried pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45 µm, it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the

## EP 1 130 042 A2

precipitate was recovered followed by vacuum drying to obtain PHA.

[0145] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 13, it was confirmed that the present PHA is PHA containing 3HBzHp as monomer units.

Table 13

Production of Polyhydroxyalkanoates by Strain H45	
Dry Weight of Cell (mg/L)	830
Weight of Polymer (mg/L)	160
Monomer Unit Composition (peak area ratio)	
3-hydroxyhexanoic acid	0.6%
3-hydroxyoctanoic acid	6.2%
3-hydroxynonanoic acid	0.1%
3-hydroxydecanoic acid	6.1%
3-hydroxydodecanoic acid	1.4%
3-hydroxydodecenoic acid	1.7%
3-hydroxy-5-benzoylvaleric acid	38.9%
3-hydroxy-7-benzoylheptanoic acid	45.0%

[Example 11]

[0146] *Pseudomonas jessenii* strain P161 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHpA followed by being cultured with shaking at 30°C at 125 strokes/min. After 47 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzHpA without containing a nitrogen source (NH<sub>4</sub>Cl), followed by being cultured with shaking at 30°C at 125 strokes/min. After 43 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and lyophilized.

[0147] This lyophilized pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45 μm, it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

[0148] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 14, it was confirmed that the present PHA is PHA containing 3HBzHp as monomer units.

Table 14

Production of Polyhydroxyalkanoates by Strain P161	
Dry Weight of Cell (mg/L)	1000
Weight of Polymer (mg/L)	420
Monomer Unit Composition (peak area ratio)	
3-hydroxybutyric acid	0.2%
3-hydroxyhexanoic acid	0.9%
3-hydroxyoctanoic acid	9.0%
3-hydroxydecanoic acid	13.7%
3-hydroxydodecanoic acid	3.2%
3-hydroxydodecenoic acid	5.0%
3-hydroxy-5-benzoylvaleric acid	29.4%
3-hydroxy-7-benzoylheptanoic acid	38.6%

[Example 12]

[0149] *Pseudomonas cichorii* strain YN2 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzOA followed by being cultured with shaking at 30°C at 125 strokes/min. After 47 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzOA without containing a nitrogen source (NH<sub>4</sub>Cl), followed by being cultured with shaking at 30°C at 125 strokes/min. After 43 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and vacuum-dried.

[0150] This vacuum-dried pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extract solution through a membrane filter with the pore size of 0.45 µm, it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

[0151] The obtained PHA was analyzed in the following measurement conditions using the NMR spectrometer (FT-NMR: Bruker DPX400).

<Measurement Conditions>

[0152]

Measuring nuclide: <sup>1</sup>H

Solvent used: CDCl<sub>3</sub>

(TMS/CDCl<sub>3</sub> sealed with a capillary was used as a reference)

Resonance frequency: <sup>1</sup>H = 400 MHz

The <sup>1</sup>H-NMR spectra are shown in FIG. 6.

[0153] From the <sup>1</sup>H-NMR spectra shown in FIG. 6, it was found that the PHA having monomer units represented by the above-described Chemical Formula [6] was obtained.

[0154] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. The results are shown in Table 15.

Table 15

Production of Polyhydroxyalkanoates by Strain YN2	
Dry Weight of Cell (mg/L)	710
Weight of Polymer (mg/L)	170
Monomer Unit Composition (peak area ratio)	
3-hydroxybutyric acid	0.1%
3-hydroxyhexanoic acid	0.4%
3-hydroxyoctanoic acid	3.9%
3-hydroxynonanoic acid	0.1%
3-hydroxydecanoic acid	3.6%
3-hydroxydodecanoic acid	1.6%
3-hydroxydodecenoic acid	3.1%
3-hydroxytetradecanoic acid	0.2%
3-hydroxy-5-phenylvaleric acid	0.2%
3-hydroxy-6-benzoylhexanoic acid	37.8%
3-hydroxy-8-benzoyloctanoic acid	49.0%

[0155] From the above results, it was confirmed that the present PHA is PHA containing 3HBzO as monomer units.

[0156] The molecular weight of the PHA was evaluated by Gel Permeation Chromatography (GPC; Tosoh HLC-8020, column; Polymer Laboratory PL gel MIXED-C (5 µm), solvent; chloroform, converted into polystyrene), thereby obtaining Mn = 41,000 and Mw = 93,000.

[Example 13]

[0157] *Pseudomonas cichorii* strain H45 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose

and 0.1% BzOA followed by being cultured with shaking at 30°C at 125 strokes/min. After 47 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzOA without containing a nitrogen source (NH<sub>4</sub>Cl), followed by being cultured with shaking at 30°C at 125 strokes/min. After 43 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and vacuum-dried.

[0158] This vacuum-dried pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extracted solution through a membrane filter with the pore size of 0.45 µm, it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

[0159] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 16, it was confirmed that the present PHA is PHA containing 3HBzO as monomer units.

Table 16

Production of Polyhydroxyalkanoates by Strain H45	
Dry Weight of Cell (mg/L)	720
Weight of Polymer (mg/L)	160
Monomer Unit Composition (peak area ratio)	
3-hydroxyhexanoic acid	0.6%
3-hydroxyoctanoic acid	4.9%
3-hydroxydecanoic acid	3.3%
3-hydroxydodecanoic acid	1.9%
3-hydroxydodecenoic acid	1.1%
3-hydroxy-6-benzoylhexanoic acid	21.3%
3-hydroxy-8-benzoyloctanoic acid	66.9%

[Example 14]

[0160] *Pseudomonas jessenii* strain P161 was inoculated into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzOA followed by being cultured with shaking at 30°C at 125 strokes/min. After 47 hr, the cells were recovered by centrifugal separation, then resuspended into 200 mL of the M 9 medium containing 0.5% D-glucose and 0.1% BzOA without containing a nitrogen source (NH<sub>4</sub>Cl), followed by being cultured with shaking at 30°C at 125 strokes/min. After 43 hr, the cells were recovered by centrifugal separation, cleaned once with cold methanol and lyophilized.

[0161] This lyophilized pellet was suspended into 20 mL chloroform and stirred at 60°C for 20 hr, and PHA was extracted. After filtrating the extract solution through a membrane filter with the pore size of 0.45 µm, it was concentrated with a rotary evaporator, and the concentrated solution was reprecipitated in cold methanol, further only the precipitate was recovered followed by vacuum drying to obtain PHA.

[0162] After performing methanolysis of the obtained PHA according to the conventional method, it was analyzed using a gas chromatography-mass spectrometer (GC-MS, Shimadzu QP-5050, EI method) to identify the methyl ester compound of PHA monomer units. As a result, as shown in Table 17, it was confirmed that the present PHA is PHA containing 3HBzO as monomer units.

Table 17

Production of Polyhydroxyalkanoates by Strain P161	
Dry Weight of Cell (mg/L)	820
Weight of Polymer (mg/L)	140
Monomer Unit Composition (peak area ratio)	
3-hydroxybutyric acid	0.9%
3-hydroxyhexanoic acid	1.4%
3-hydroxyoctanoic acid	11.5%
3-hydroxynonanoic acid	0.1%
3-hydroxydecanoic acid	8.9%
3-hydroxydodecanoic acid	1.8%

**EP 1 130 042 A2**

Table 17 (continued)

Monomer Unit Composition (peak area ratio)	
3-hydroxydodecenoic acid	3.3%
3-hydroxytetradecanoic acid	0.3%
3-hydroxy-6-benzoylhexanoic acid	23.1%
3-hydroxy-8-benzoyloctanoic acid	48.7%

## SEQUENCE LISTING

5

&lt;110&gt; Canon Inc.

10

&lt;120&gt; Polyhydroxyalkanoate containing 3-hydroxybenzoylalkanoic acid as monomer unit, and method for producing the same.

15

&lt;130&gt; 4396021

20

&lt;160&gt; 1

25

&lt;210&gt; 1

&lt;211&gt; 1501

&lt;212&gt; DNA

30

&lt;213&gt; Pseudomonas jessenii 161 strain.

35

&lt;400&gt; 1

tgaacgcctgg cggcaggcct aacacatgca agtcgagcgg atgacgggag ctgtctcctg 60

40

aatlcagcgg cggacgggtg aglaatgcct aggaatctgc ctggtagtgg gggacaacgt 120

ctcgaaaggg acgctaatac cgcatacgtc ctacgggaga aagcagggga ccttcgggcc 180

ttgcgclatc agatgagcct aggtcggatt agctagltgg tgagglaatg gctcaccaag 240

45

gcgacgatcc gtaaciggtc tgagaggatg atcagtcaca ctggaactga gacacgggcc 300

agactcctac gggaggcagc agtggggaat attggacaat gggcgaaagc ctgatccagc 360

50

catgccgcgt gtgtgaagaa ggctctcgga ttglaaagca ctttaagltg ggaggaaggg 420

cattaacctt atacgttagt gttttgacgt taccgacaga ataagcaccg gctaactctg 480

tgccagcagc cgcgglaata cagagggtgc aagcgttaat cggaattact gggcgtaaag 540

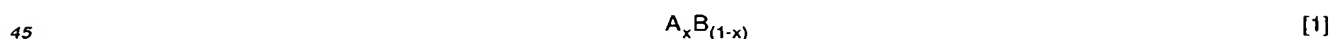
55



cgcgcgtagg tgglllgta agllggatgt gaaagccccc ggctcaacct gggaacigca 600  
 5 llcaaaaacig acaagctaga glatggtaga gggtaggtaga atttccctgig tagcggtaga 660  
 atgcgtagat ataggaagga acaccagigg cgaaggcgac caccaggact galactgaca 720  
 ctgaggigcg aaagcgggg gagcaaacag gallagatag cctggtagic cacgccglaa 780  
 10 acgatgicaa ctagccgttg ggagccctga gctcttagig gcgcagctaa cgcaatlaagi 840  
 tgaccgccig gggagtagcg ccgcaagggt aaaactcaaa tgaattgacg gggggcccgca 900  
 15 caagcggigg agcatgigg ttaattcgaa gcaacgcgaa gaacctacc aggccttagc 960  
 atccaatgaa ctctccagag atggatgggt gccttcggga acattgagac aggtgcigca 1020  
 20 tggctgtcgt cagctcgtgt cgtgagatgt tgggtlaagt cccglaacga gcgcaacctt 1080  
 tgtccctagt taccagcacg taatggtagg cactctlaagg agactgccgg tgacaaaccg 1140  
 gaggaaggig gggatgacgt caagtcacga tggcccttac ggctggggt acacacgtgc 1200  
 25 tacaatggic ggtacagagg gttgccaagc cgcgaggttg agctaatccc aaaaaaccga 1260  
 tctagtagcg gatcgagtc tgcaactcga ctgcgtgaag tcggaatcgc tagtaatcgc 1320  
 30 gaatcagaat gtcgcggtga atacgttccc gggccttgta cacaccgcc gtcacacat 1380  
 gggagtaggt tgcaccagaa gtagctagtc taaccttcgg gaggacgggt accacgggtg 1440  
 gatcaltgac tggggtagaag tctaccaag gtagccgtag gggaacctgc ggctggatca 1500  
 35 c 1501

#### 40 Claims

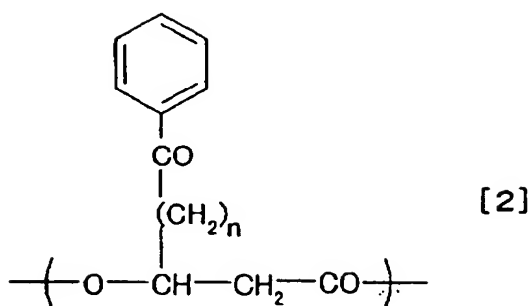
1. A polyhydroxyalkanoate having a monomer unit composition represented by the following Formula [1]:



wherein A is at least one monomer unit represented by the following Chemical Formula [2]:

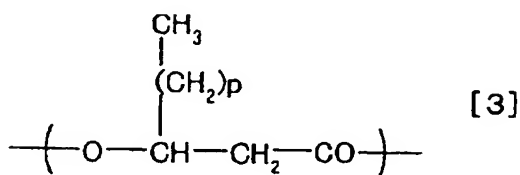
50

55

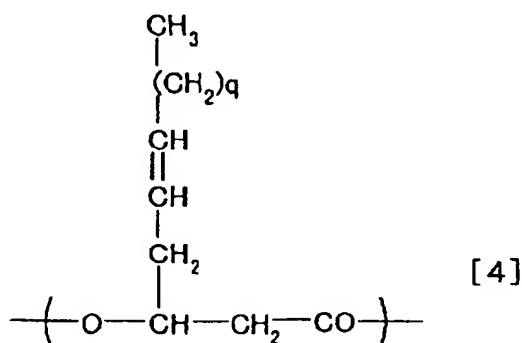


15 where n is an integer of 1 to 8; and

B is at least one selected from monomer units represented by the following Chemical Formula [3]:



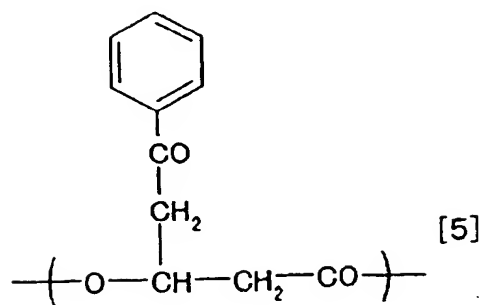
where p is an integer of 0 to 10, and monomer units represented by the following Chemical Formula [4]:



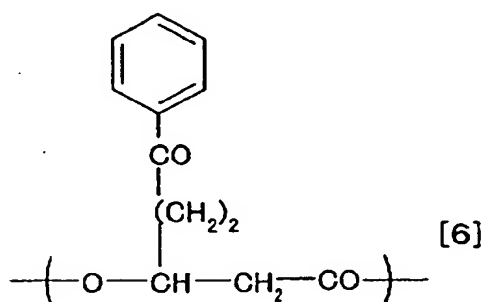
45 where q is 3 or 5; and

x is not less than 0.01 and less than 1.

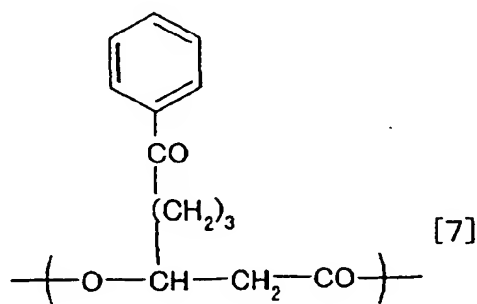
- 50
- 55
2. The polyhydroxyalkanoate according to claim 1, wherein the one or more monomer units include a monomer unit represented by the Chemical Formula [5]:



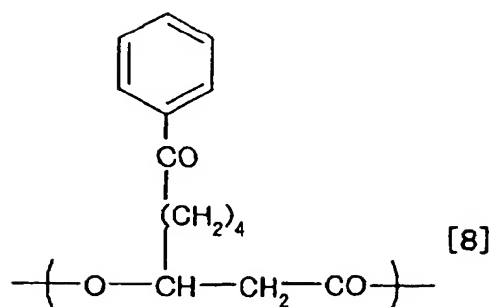
- 15 3. The polyhydroxyalkanoate according to claim 1, wherein the one or more monomer units include a monomer unit represented by the Chemical Formula [6]:



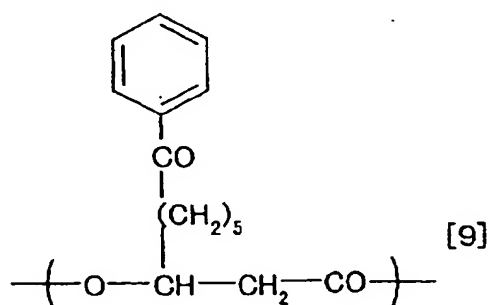
- 30 4. The polyhydroxyalkanoate according to claim 1, wherein the one or more monomer units include a monomer unit represented by the Chemical Formula [7]:



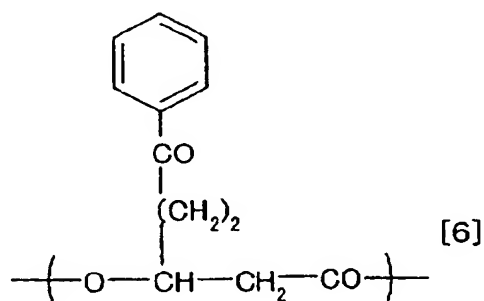
- 45 5. The polyhydroxyalkanoate according to claim 1, wherein the one or more monomer units include a monomer unit represented by the Chemical Formula [8]:
- 50
- 55



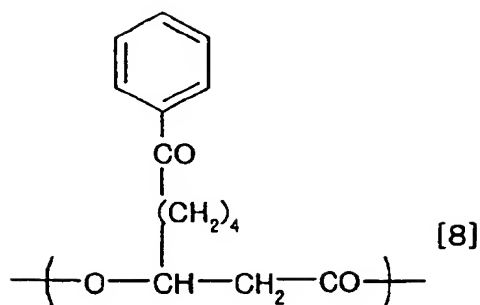
- 15 6. The polyhydroxyalkanoate according to claim 1, wherein the one or more monomer units include a monomer unit represented by the Chemical Formula [9]:



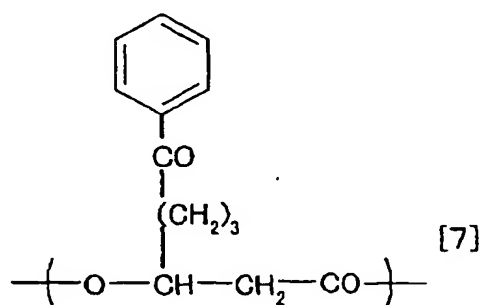
- 30 7. The polyhydroxyalkanoate according to claim 1, wherein the monomer units include a monomer unit represented by the Chemical Formula [6]:



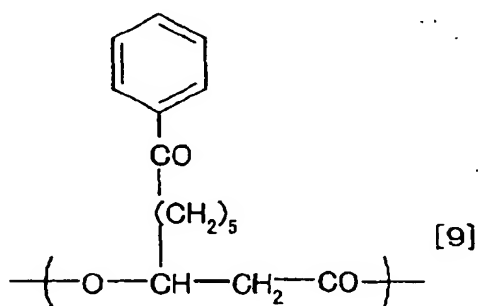
45 and a monomer unit represented by the Chemical Formula [8]:



- 15 8. The polyhydroxyalkanoate according to claim 1, wherein the monomer units include a monomer unit represented by the Chemical Formula [7]:



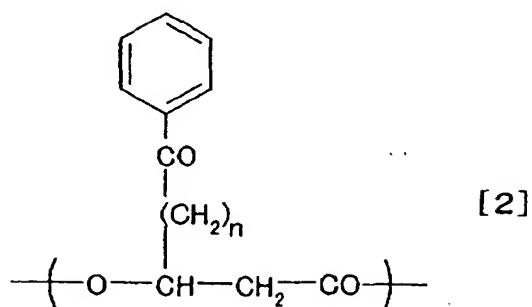
30 and a monomer unit represented by the Chemical Formula [9]:



- 45 9. The polyhydroxyalkanoate according to any one of claims 1 to 8, wherein the polyhydroxyalkanoate has a number-average molecular weight of 10,000 to 1,000,000.
- 50 10. A method for producing a polyhydroxyalkanoate, comprising the step of culturing, in a medium containing a benzoylalkanoic acid, a microorganism capable of synthesizing a polyhydroxyalkanoate having a monomer unit composition represented by the following Formula [1]:

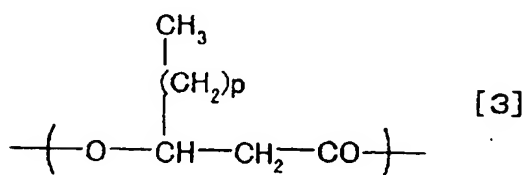


55 from the benzoylalkanoic acid by utilizing the benzoylalkanoic acid, wherein A is at least one monomer unit represented by the following Chemical Formula [2]:

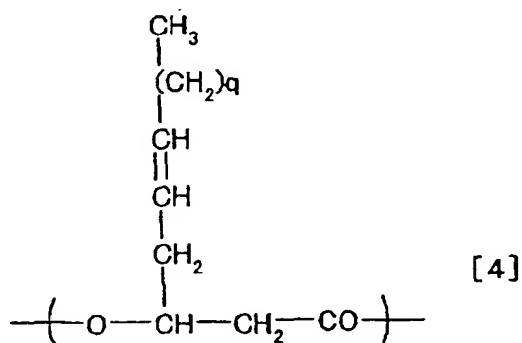


where n is an integer not less than 1;

B is at least one selected from monomer units represented by the following Chemical Formula [3]:



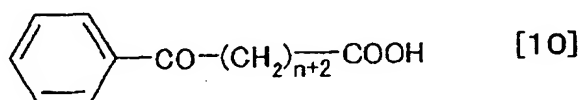
where p is an integer of 0 to 10, and monomer units represented by the following Chemical Formula [4]:



where q is 3 or 5; and

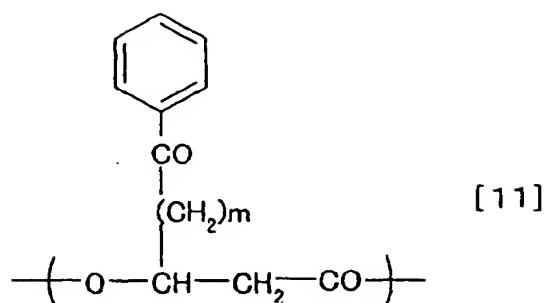
x is not less than 0.01 to less than 1.

- 45 **11.** The method according to claim 10, wherein the benzoylalkanoic acid is a benzoylalkanoic acid represented by the following Chemical Formula [10]:



where n is an integer of 1 to 8, and

the polyhydroxyalkanoate is a polyhydroxyalkanoate comprising a monomer unit represented by the following Chemical Formula [11]:



15 where m is at least one or more selected from the group consisting of n, n-2, n-4 and n-6, and is an integer not less than 1.

20 12. The method according to claim 10, wherein the step of culturing the microorganism is a step of culturing the microorganism capable of producing the polyhydroxyalkanoate having the monomer unit composition represented by the Formula [1] in a medium containing the benzoylalkanoic acid and a saccharide, by utilizing the benzoylalkanoic acid.

13. The method according to claim 12, wherein the culture of the microorganism is performed in one step by using a medium containing the benzoylalkanoic acid and the saccharide.

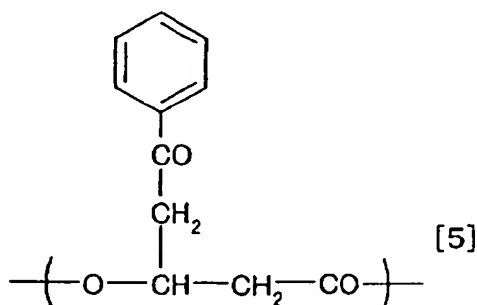
25 14. The method according to claim 12, wherein the culture of the microorganism is performed in at least two steps by culturing in a medium containing the benzoylalkanoic acid and the saccharide and then culturing in a medium containing the benzoylalkanoic acid and the saccharide in which a nitrogen source is limited.

30 15. The method according to claim 12, wherein the saccharide is at least one selected from the group consisting of glucose, fructose and mannose.

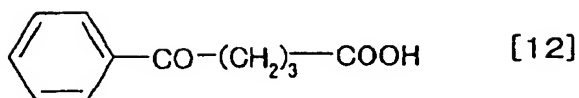
16. The method according to any one of claims 10 to 12, wherein the microorganism is a microorganism belonging to *Pseudomonas* sp.

35 17. The method according to claim 16, wherein the microorganism is at least one strain selected from the group consisting of *Pseudomonas cichorii* H45, FERM BP-7374; *Pseudomonas cichorii* YN2, FERM BP-7375; and *Pseudomonas jessenii* P161, FERM BP-7376.

40 18. The method according to any one of claims 10 to 12, wherein the step of culturing the microorganism is a step of culturing a microorganism capable of producing a polyhydroxyalkanoate comprising a monomer unit represented by the following Chemical Formula [5]:

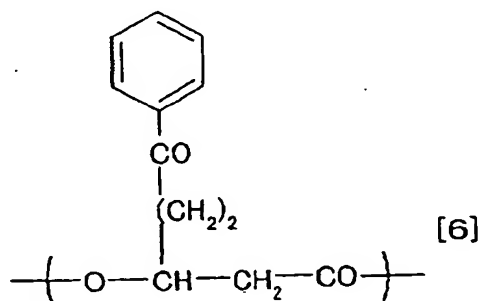


in a medium containing 4-benzoylbutyric acid represented by the following Chemical Formula [12]:

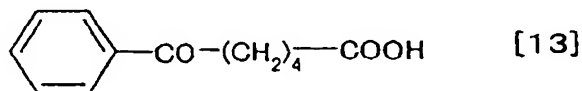


by utilizing 4-benzoylbutyric acid; and wherein the produced polyhydroxyalkanoate comprises a monomer unit represented by the Chemical Formula [5].

19. The method according to any one of claims 10 to 12, wherein the step of culturing the microorganism is a step of culturing a microorganism capable of producing a polyhydroxyalkanoate comprising a monomer unit represented by the following Chemical Formula [6]:

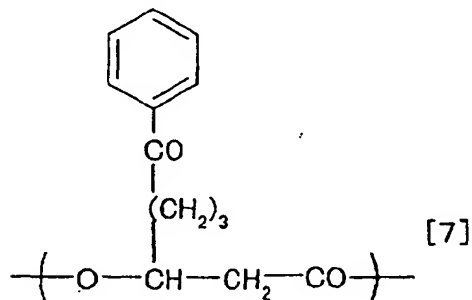


in a medium containing 5-benzoylvaleric acid represented by the following Chemical Formula [13]:



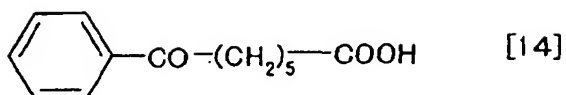
by utilizing 5-benzoylvaleric acid, and wherein the produced polyhydroxyalkanoate comprises a monomer unit represented by the Chemical Formula [6].

20. The method according to any one of claims 10 to 12, wherein the step of culturing the microorganism is a step of culturing a microorganism capable of producing a polyhydroxyalkanoate comprising a monomer unit represented by the following Chemical Formula [7]:



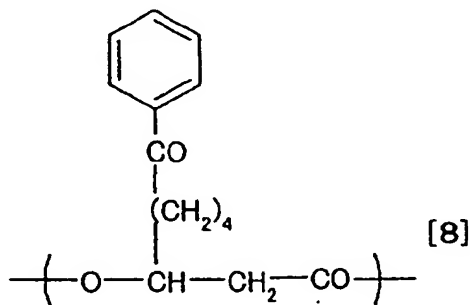
in a medium containing 6-benzoylhexanoic acid represented by the following Chemical Formula [14]:



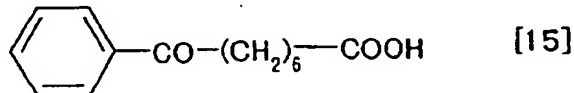


by utilizing 6-benzoylhexanoic acid, and wherein the produced polyhydroxyalkanoate comprises a monomer unit represented by the Chemical Formula [7].

21. The method according to any one of claims 10 to 12, wherein the step of culturing the microorganism is a step of culturing a microorganism capable of producing a polyhydroxyalkanoate comprising a monomer unit represented by the following Chemical Formula [8]:

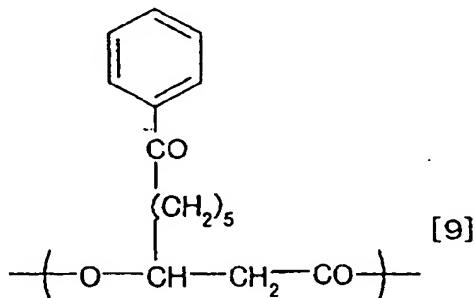


in a medium containing 7-benzoylheptanoic acid represented by the following Chemical Formula [15]:

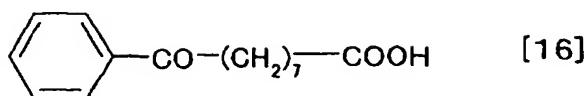


by utilizing 7-benzoylheptanoic acid, and wherein the produced polyhydroxyalkanoate comprises a monomer unit represented by the Chemical Formula [8].

22. The method according to any one of claims 10 to 12, wherein the step of culturing the microorganism is a step of culturing a microorganism capable of producing a polyhydroxyalkanoate comprising a monomer unit represented by the following Chemical Formula [9]:

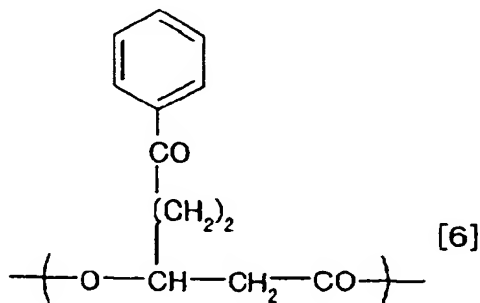


in a medium containing 8-benzoyloctanoic acid represented by the following Chemical Formula [16]:

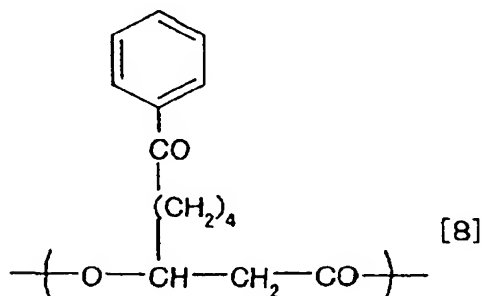


by utilizing 8-benzoyloctanoic acid, and wherein the produced polyhydroxyalkanoate comprises the monomer unit represented by the Chemical Formula [9].

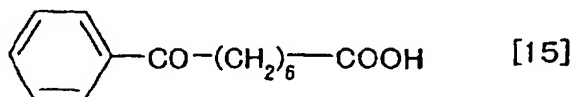
23. The method according to any one of claims 10 to 12, wherein the step of culturing the microorganism is a step of culturing a microorganism capable of producing a polyhydroxyalkanoate comprising monomer units represented by the following Chemical Formula [6]:



and the following Chemical Formula [8]:



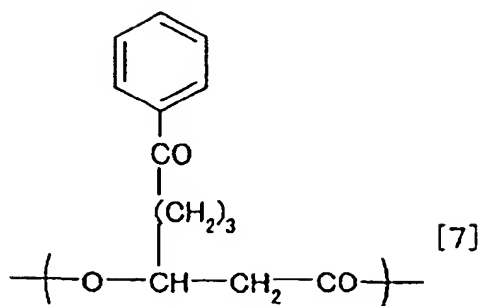
in a medium containing 7-benzoylheptanoic acid represented by the following Chemical Formula [15]:



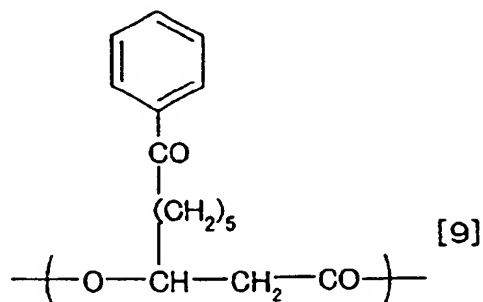
by utilizing 7-benzoylheptanoic acid, and wherein the produced polyhydroxyalkanoate comprises monomer units represented by the Chemical Formulas [6] and [8].

24. The method according to any one of claims 10 to 12, wherein the step of culturing the microorganism is a step of culturing a microorganism capable of producing a polyhydroxyalkanoate comprising monomer units represented

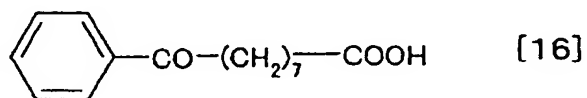
by the following Chemical Formula [7]:



and the following Chemical Formula [9]:



in a medium containing 8-benzoyloctanoic acid represented by the following Chemical Formula [16]:



by utilizing 8-benzoyloctanoic acid, and wherein the produced polyhydroxyalkanoate comprises monomer units represented by the Chemical Formulas [7] and [9].

FIG. 1

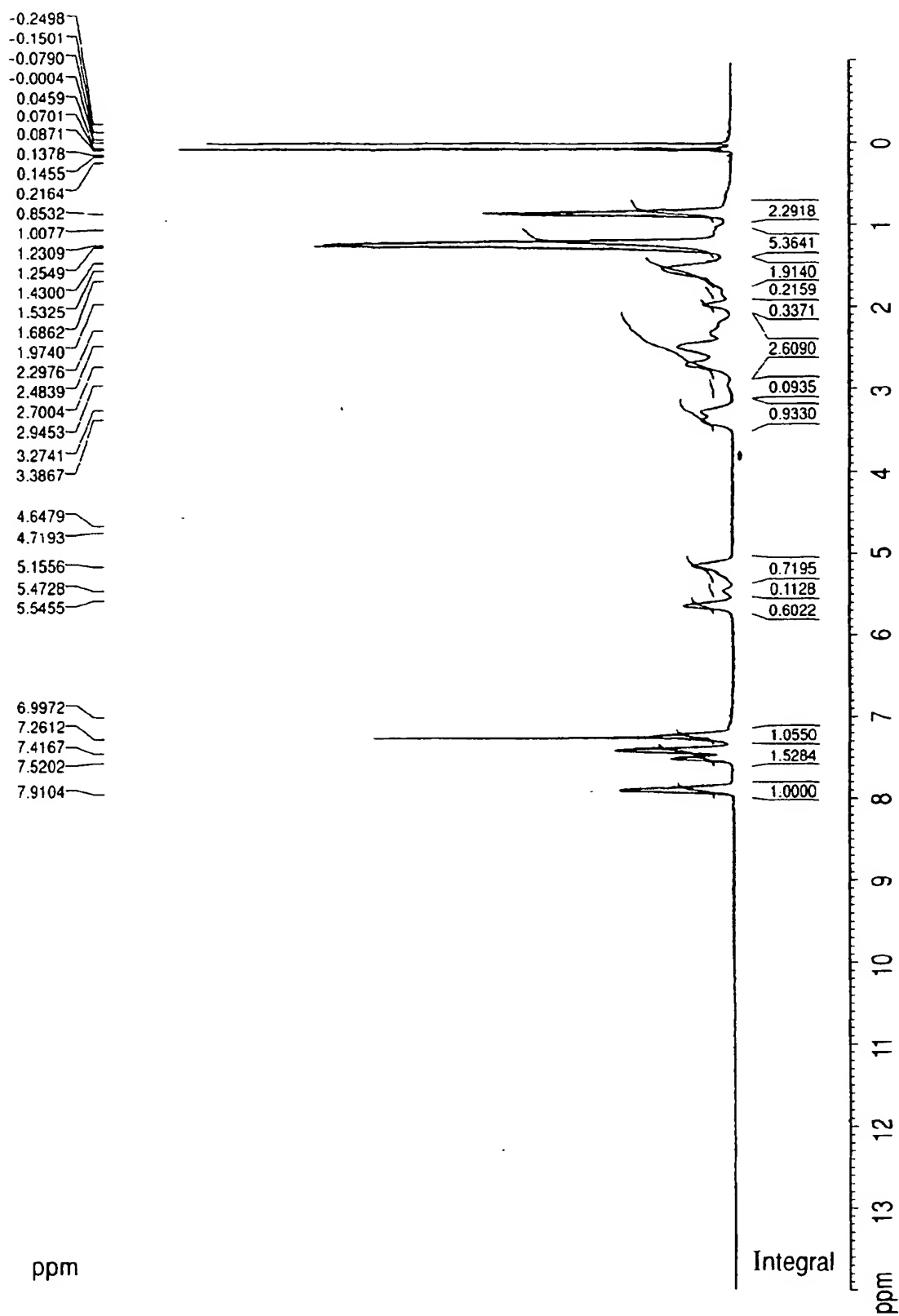


FIG. 2

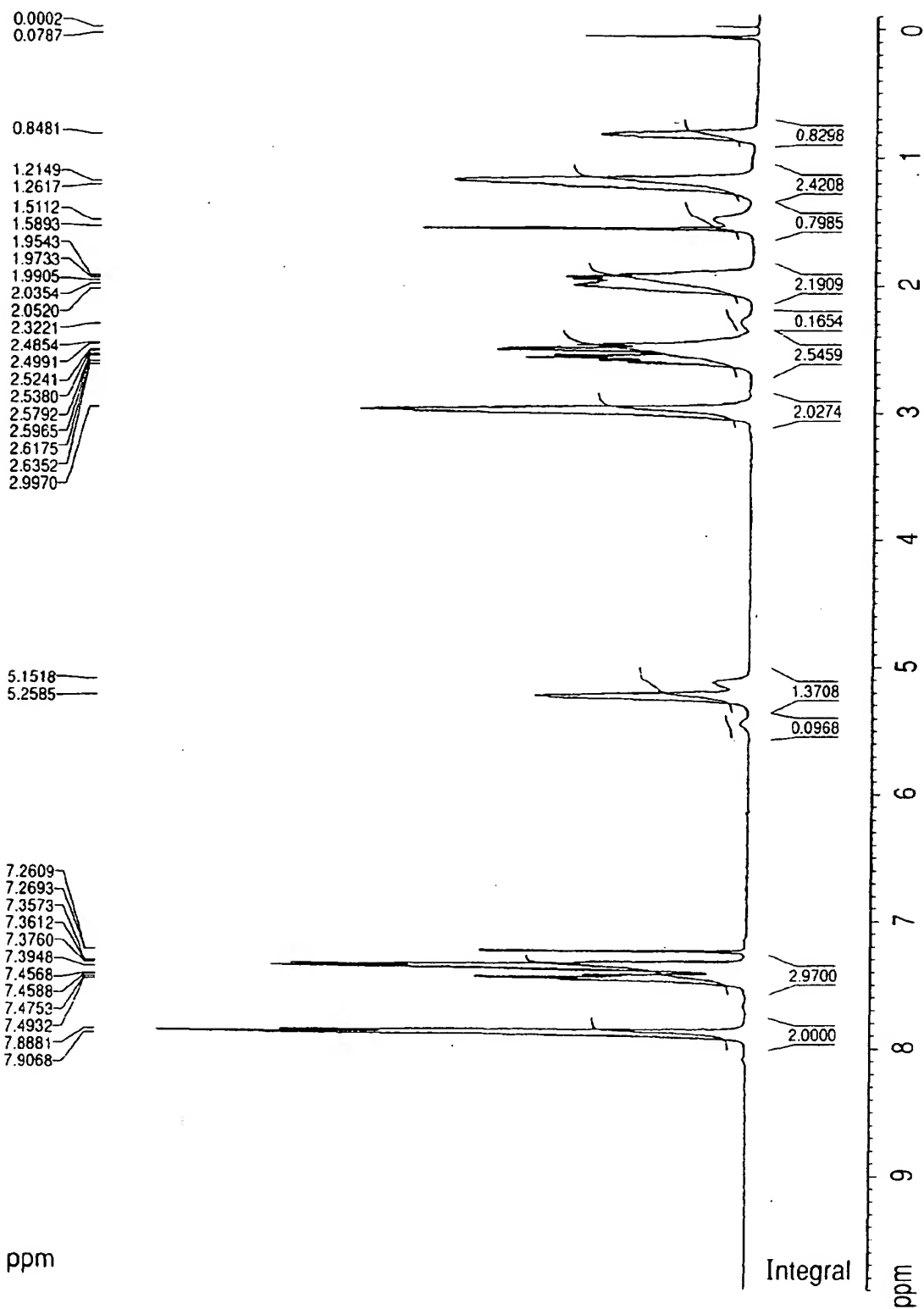


FIG. 3

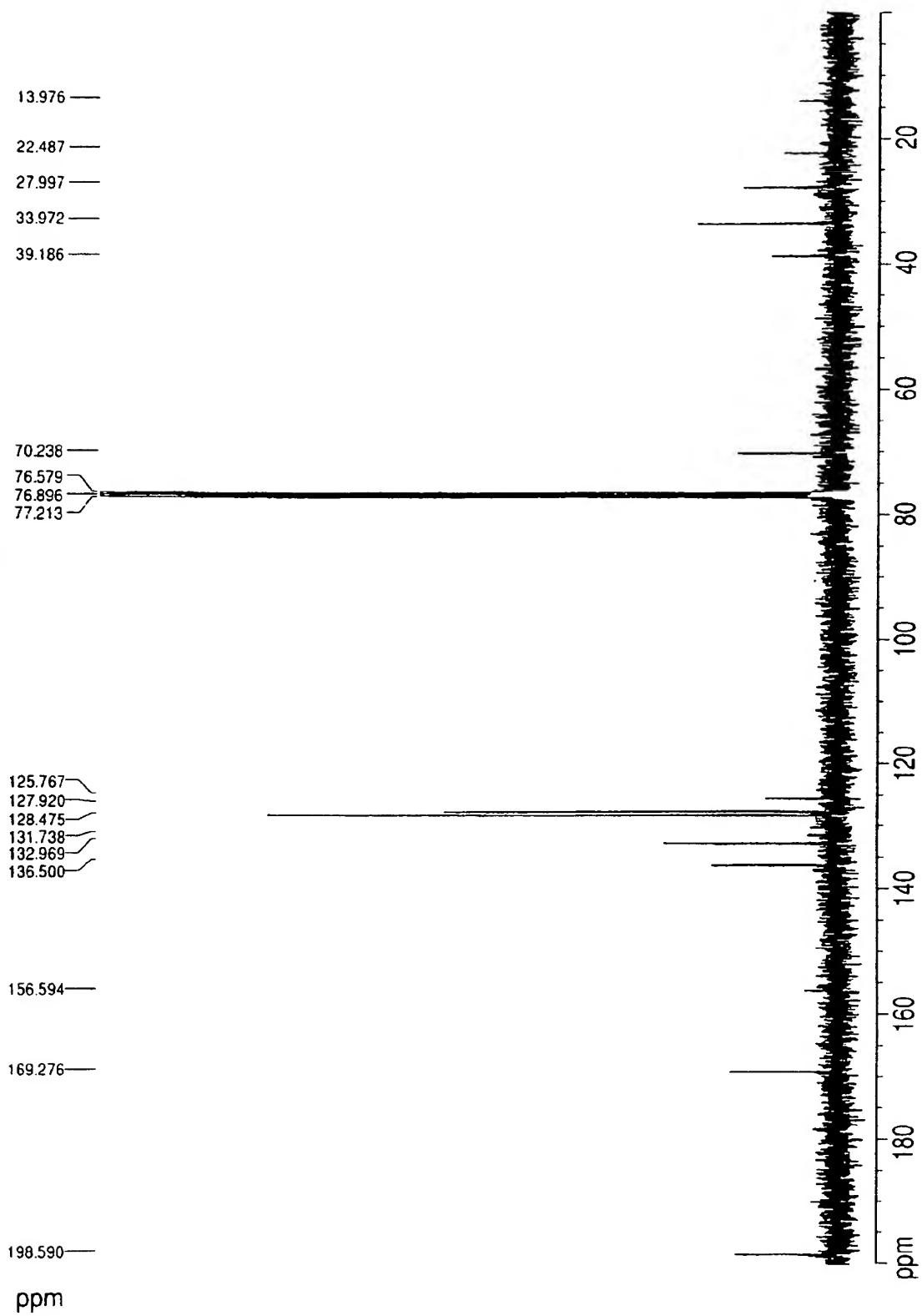


FIG. 4

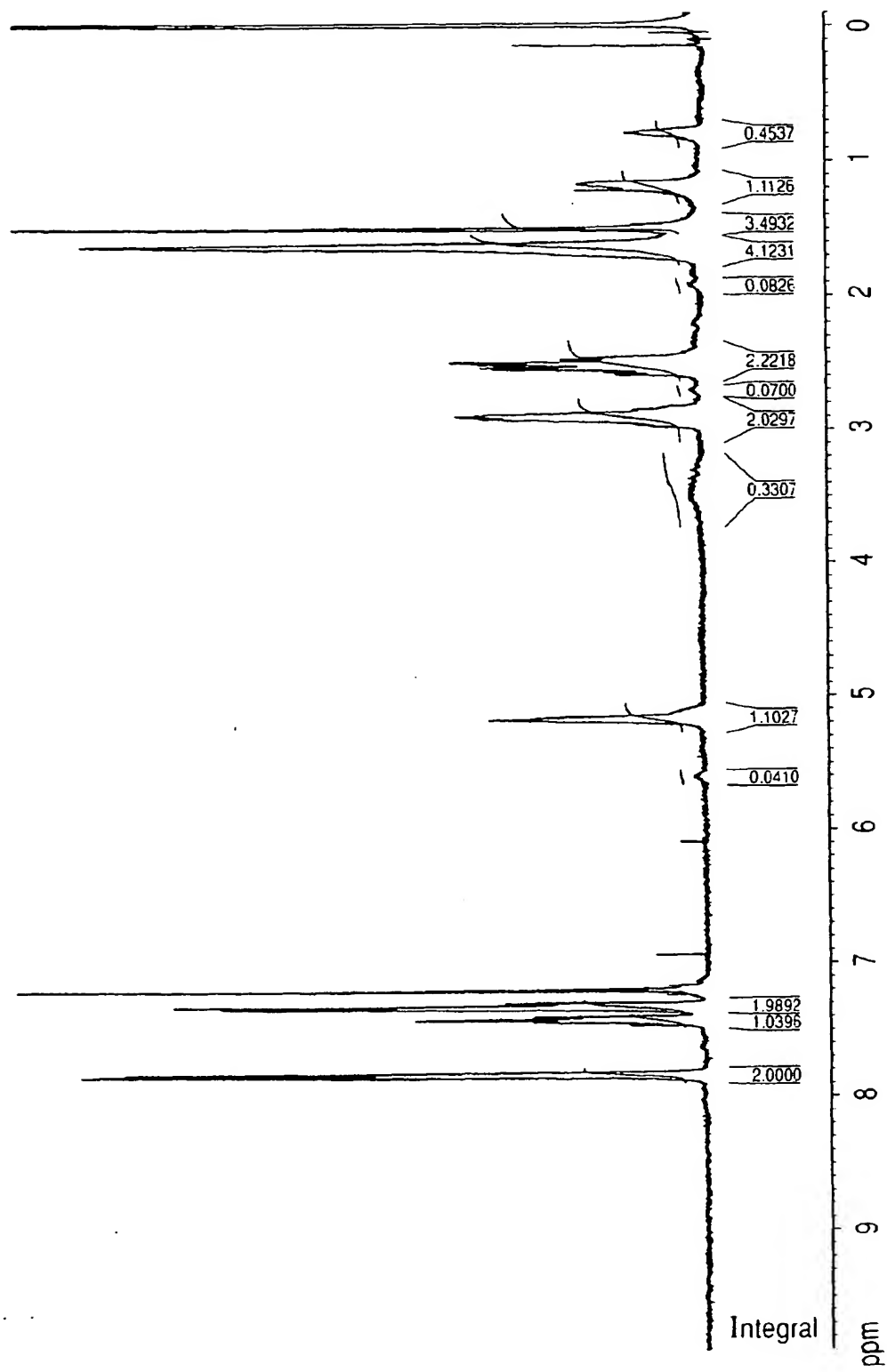


FIG. 5

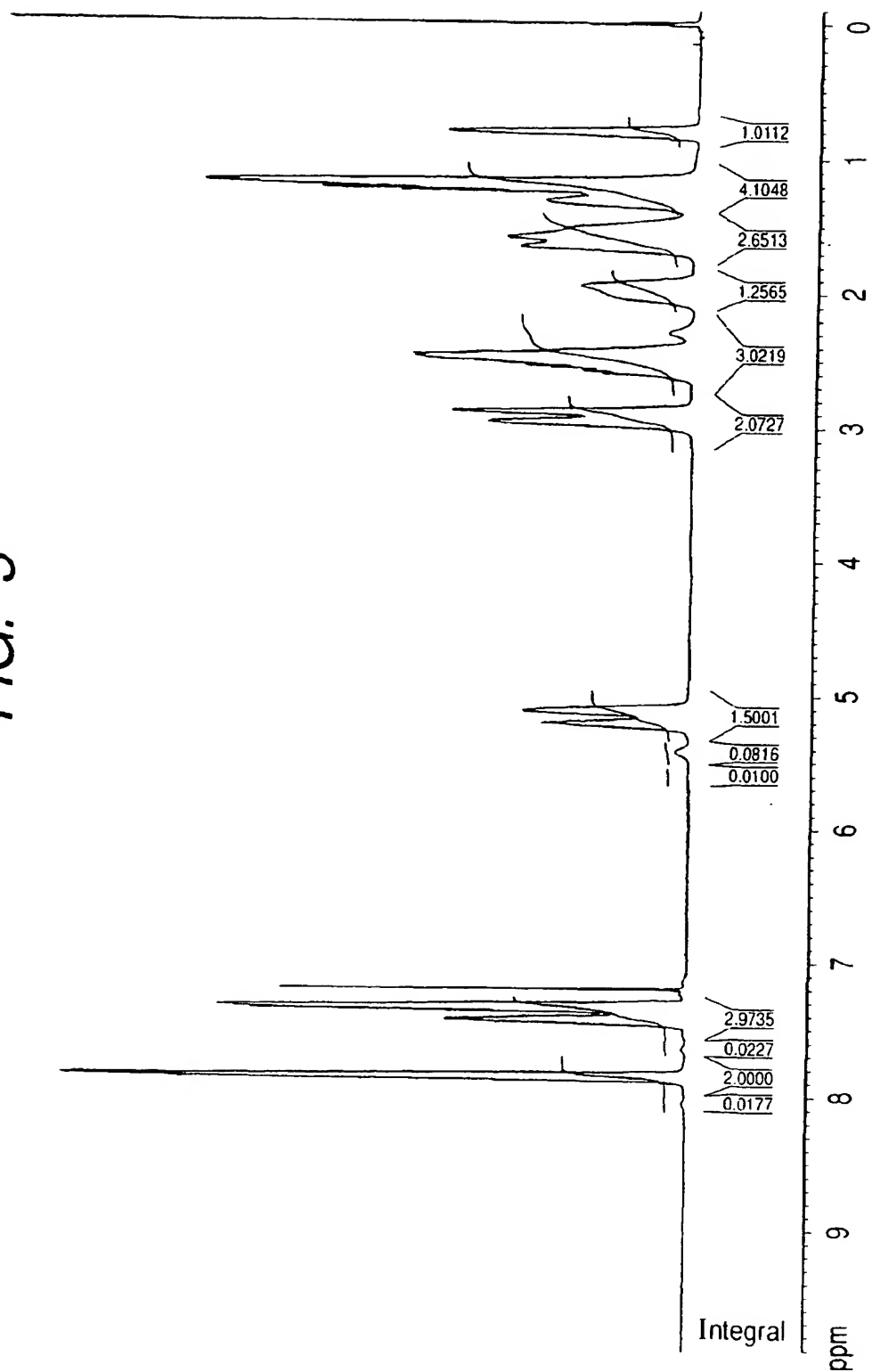
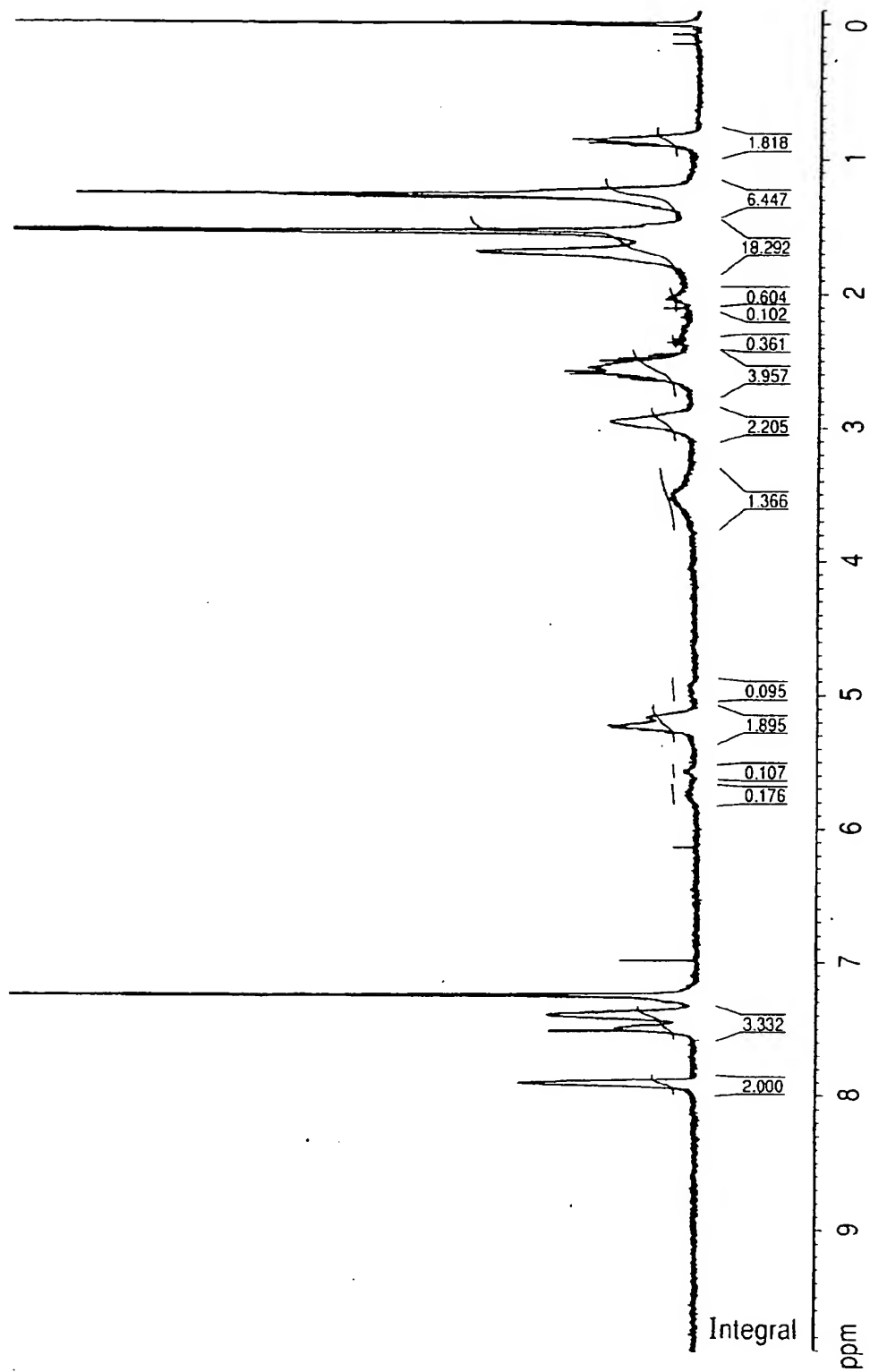




FIG. 6



***This Page Blank (uspto)***





European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 01 10 4925

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A, D	FRITZSCHE K ET AL: "AN UNUSUAL BACTERIAL POLYESTER WITH A PHENYL PENDANT GROUP" MAKROMOLEKULARE CHEMIE, MACROMOLECULAR CHEMISTRY AND PHYSICS, HUTHIG UND WEPF VERLAG, BASEL, CH, vol. 191, 1990, pages 1957-1965, XP000985669 ISSN: 0025-116X * the whole document *	1-24	C08G63/06 C12P7/62
A, D	CURLEY J M ET AL: "PRODUCTION OF POLY(3-HYDROXYALKANOATES) CONTAINING AROMATIC SUBSTITUENTS BY PSEUDOMONAS OLEOVORANS" MACROMOLECULES, AMERICAN CHEMICAL SOCIETY, EASTON, US, vol. 29, no. 5, 26 February 1996 (1996-02-26), pages 1762-1766, XP000555551 ISSN: 0024-9297 * the whole document *	1-24	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	EP 0 392 687 A (ICI PLC) 17 October 1990 (1990-10-17) * the whole document *	1-24	C08G C12P C12N
A	US 4 477 654 A (HOLMES PAUL A ET AL) 16 October 1984 (1984-10-16) * the whole document *	1-24	
A	EP 0 288 908 A (MITSUBISHI GAS CHEMICAL CO) 2 November 1988 (1988-11-02) * the whole document *	1-24	
		-/--	
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 6 May 2002	Examiner Masson, P
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

EPO FORM 1503 03/92 (P/CU1)



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number  
EP 01 10 4925

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A,D	<p>KIM O ET AL: "BIOENGINEERING OF POLY(BETA-HYDROXYALKANOATES) FOR ADVANCED MATERIAL APPLICATIONS: INCORPORATION OF CYANO AND NITROPHENOXY SIDECHAIN SUBSTITUENTS"</p> <p>CANADIAN JOURNAL OF MICROBIOLOGY, OTTAWA, CA, vol. 41, no. SUPPL 1, 1995, pages 32-43, XP000985628 ISSN: 0008-4166 * the whole document *</p> <p>-----</p>	1-24	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
The present search report has been drawn up for all claims			
Place of search <b>MUNICH</b>		Date of completion of the search <b>6 May 2002</b>	Examiner <b>Masson, P</b>
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p>		<p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>&amp; : member of the same patent family, corresponding document</p>	

EPO FORM 1503 03.82 (Pdc01)

**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 01 10 4925

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

06-05-2002

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
EP 0392687	A	17-10-1990	AT	126538 T	15-09-1995
			AU	634218 B2	18-02-1993
			AU	5240590 A	18-10-1990
			BR	9001732 A	04-06-1991
			CA	2014238 A1	12-10-1990
			DE	69021613 D1	21-09-1995
			DE	69021613 T2	08-02-1996
			EP	0392687 A2	17-10-1990
			JP	2299590 A	11-12-1990
			MW	2390 A1	14-11-1990
			NZ	233296 A	28-10-1992
			US	5387513 A	07-02-1995
			US	5296362 A	22-03-1994
			ZA	9002399 A	27-02-1991
US 4477654	A	16-10-1984	AU	560653 B2	16-04-1987
			AU	8410882 A	13-01-1983
			DE	3173154 D1	16-01-1986
			DE	3276072 D1	21-05-1987
			EP	0052459 A1	26-05-1982
			EP	0069497 A2	12-01-1983
			JP	1956516 C	10-08-1995
			JP	4038763 B	25-06-1992
			JP	58069224 A	25-04-1983
			JP	2008865 C	11-01-1996
			JP	6172501 A	21-06-1994
			JP	7047623 B	24-05-1995
			JP	2577841 B2	05-02-1997
			JP	5015383 A	26-01-1993
			NZ	201054 A	08-11-1985
			SU	1375143 A3	15-02-1988
EP 0288908	A	02-11-1988	JP	1728448 C	19-01-1993
			JP	4012712 B	05-03-1992
			JP	63269989 A	08-11-1988
			JP	1069622 A	15-03-1989
			JP	1727770 C	19-01-1993
			JP	4012713 B	05-03-1992
			CA	1338142 A1	12-03-1996
			DE	3875367 D1	26-11-1992
			DE	3875367 T2	25-03-1993
			EP	0288908 A2	02-11-1988
			US	4997909 A	05-03-1991

EPO FORM P449

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☒ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**This Page Blank (uspto)**